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A STUDY OF AETIOLOGICAL FACTORS IN ORAL SQUAMOUS CELL CARCINOMA

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It is a rather intriguing fact that the organ incidence of malignant disease varies strikingly in different regions of the world and in different races. Each country appears to have its own dominant cancer problem. In India oral and pharyngeal cancers are by far the commonest. Next comes carcinoma of the uterine cervix. This is true of all India. Oro-pharyngeal carcinomas constitute 48 per cent of all malignancies at the Cancer Institute, Madras. The Tata Memorial Hospital figures indicate a frequency of 49 per cent of oro-pharyngeal carcinomas of all malignancies seen (Khanolkar). The reason for this peculiarly high incidence of oro-pharyngeal carcinomas in India must bear a relationship to either the mode of life, the habits and customs of the people or a distinctive environment in which they live or to the genetic constitution of the races.

Indians are of multiracial descent and it is not likely that the different races in India have the same genetic constitutional susceptibility to oral cancers when even individual susceptibility varies so widely. We thought it reasonable to look for possible aetiological factors in the environment, possible specific exposures to carcinogens in the food, in the habits and customs and the occupations of the people.

MATERIAL

The oral and pharyngeal cancers that were studied were those of the mucosa of the cheek, the lip, the floor of the mouth and lower gingivum, the palate and upper gingivum, the anterior two-thirds and the posterior third of the tongue, the oro-pharynx, the epilarynx and the laryngopharynx, but only cancers of the buccal mucosa and the tongue are discussed here. The naso-pharyngeal carcinomas were very rare indeed and too few for study and the other pharyngeal carcinomas will be discussed elsewhere. All the carcinomas studied were squamous cell carcinomas. The total number of cases studied were 347 over a one year period. Table I gives the relative site incidence of these cancers as a percentage of all the malignancies seen.

Table II gives the site incidence of these cancers as a percentage of all the intra-oral cancers seen.

It will be obvious from the tables that 71 per cent of all oral carcinomas and 26·45 per cent of all malignancies seen at the Cancer Institute, Madras, arose from the buccal mucosa and 22 per cent of all oral cancers and 8 per cent of all malignancies from the lingual mucosa.

TABLE I.—*Incidence of Oral Cancers*

Site of cancer	Oral cancer as a percentage of all malignancies seen
Cheek	19·20
Tongue	8·23
Floor of mouth and gingivum	7·25
Palate	3·92
Lip	0·39
Tonsil	2·15
Pharynx (excluding tonsil, but including oropharynx, epilarynx, laryngopharynx)	6·86
Total	48·00

TABLE II.—*Site Incidence of Oral Cancers*

Site	Site incidence as percentage of all oral cancers seen
Cheek	51·61
Tongue	22·1
Floor of mouth and gingivum	19·5
Palate and upper gingivum	5·7
Lip	1·09

The investigation of the aetiology and the methods of control of this group of cancers has, therefore, assumed a position of prime importance in our research programmes.

Possible Aetiological Factors

An analysis of possible aetiological factors elicited the following information:—

Carcinoma of the cheek and floor of mouth including lower gingivum

The total number of carcinomas of the cheek and floor of the mouth analysed were 206. A random control group of 278 people drawn from a non-cancerous population were also screened for comparative study. Both samples were from the same age groups. The youngest patient was 22 years old and the oldest 66 years, the average age being 52·9 years. The average age of the control group was 44–45 years, the youngest being 14 years and the oldest 70 years.

The results of the study are shown in Table III.

The sex, religious and environmental distribution of the control group were taken from the Government of India Census report of 1954. The rest of the control group figures were taken from our own sample survey.

The incidence of carcinoma of the cheek is significantly higher in the male. The reason for this lies most likely in the habits of the male population rather than in the sex hormones.

There is not much significance in the religious or environmental distribution of the disease. The Government of India Census from which the control figures are taken excludes a number of subsects which are classified under separate heads. The cancer figures include the subsects. The 10 per cent difference in the environmental incidence between the cancerous population and the control population is most probably due to the urban population seeking advice in a city hospital like ours more readily.

TABLE III.—*Patients with Carcinoma of Cheek Compared with a Control Group*

Factor studied	Carcinoma cheek Group No. 206 (%)	Control Group No. 278 (%)
1. Sex—		
Male	65	50
Female	35	50
2. Religion—		
Hindus	90	84·99
Christians	5	2·5
Moslems	5	9·93
3. Social status—		
Upper middle class	0·009	Reliable data
Lower middle class	34·99	not available
Labour	65	
4. Environment—		
Rural	65	75·5
Urban	35	24·4
5. Diet—		
Vegetarian	19	20
Non-vegetarian	81	80
6. Habits—		
Betel nut and tobacco chewing	85	12·5
Betel and nut chewing	8·7	51·8
Smoking	26	47
Alcohol	17	Data not available
7. Family history—		
Cancer in the family at any site	3·8	5·7
Oral cancer in the family	2·54	Not taken
8. Pre-existing illness—		
Dental sepsis	99·9	50
Syphilis	10·6	3·57
Tubercle	0	1
Diabetes	3·8	0·7
Hypertension	2·5	0·7
Leucoplakia and stomatitis	22·5	6
Virus infection	2	0
Anaemia	10·7	1·4
9. Fractional testmeal—		
Achlorhydria	28·3	36
Hypochlorhydria	32·7	44·5
Hyperchlorhydria	8·6	0
Normal	30·4	19·5

The lower middle class population in India equals, if it does not exceed, the labour population though exact figures are not available in the Government of India Census. The fact that carcinoma of the cheek is nearly twice as frequent in labour as in the lower middle class population is, therefore, significant.

The nature of the diet does not seem to have a significant aetiological bearing. Nor does there appear to be any familial or hereditary tendency.

The fact that 85 per cent of cheek carcinoma patients were tobacco, betel and nut chewers against 12·5 per cent in the control group is very striking. Equally striking is the fact that only 8·7 per cent of cheek cancer patients were pure betel and nut chewers as against 51·8 per cent in the control group. Smoking of any kind does not appear to be of importance. Most of the patients were cheroot smokers (80 per cent). The total absence of alcohol habits in the control group should not be taken at its face value as the survey was carried out in a prohibition area and men who are not patients are not very honest in such matters.

It is a well known fact that the percentage of alcohol addicts in Madras State very easily exceeds 10 per cent.

In considering pre-existing illnesses tuberculosis, diabetes, hypertension and virus diseases can be ruled out. In considering syphilis one should remember that the disease in the cancer patients was detected by serological tests and most of the patients did not exhibit any clinical evidence or give a history of the disease. In the control survey we had to depend entirely on the history given and we are quite sure that the actual incidence would have been much higher than the revealed figure of 3·57 per cent. Taking all these points into consideration and the fact that the 10·6 per cent syphilitics in the cancer group were latent cases we did not consider that syphilis as such had any aetiological significance.

Leucoplakia is more a stage in the evolution of mucosal cancer than an aetiological factor and, therefore, cannot be considered under the latter category.

It is only to be expected that a larger number of cancer patients would be anaemic compared to a healthy population and, therefore, a frequency of 10·7 per cent anaemia in the cancer group as compared to 1·4 per cent in the controls can hardly be of any significance.

The gastric juice analysis was carried out in all the cancer patients but only in 50 individuals in the control group. The percentage of normality is lower and the percentage of abnormalities higher in the control group than in the study group. This is most probably due to the fact that the fractional test meal control group consisted of cases that attended our outpatient department for complaints other than malignant neoplasia. They were not in normal health. The analysis, however, showed that there was no particular relationship between free acidity in the gastric juice or lack of it and oral carcinoma.

We felt that the fact that gross dental sepsis was present in 99·9 per cent of the cheek carcinomas deserved further study. Was the dental sepsis just a sequel to the oral carcinoma and the difficulty in oral hygiene when a fairly large oral ulcer was present? A careful enquiry was conducted, therefore, as to the duration of the dental sepsis and whether it was really a pre-existent condition. The enquiry very definitely proved that the habit of tobacco chewing engendered dental sepsis and this was confirmed by examination of the teeth of chronic tobacco chewers in the control group. All the chronic tobacco chewers in the control group had varying degrees of dental sepsis from moderate to gross. 50 per cent of the entire control group had dental sepsis.

This analysis has only confirmed what we had always felt—that there was a specific and a very concrete relationship between the habit of tobacco, betel and nut chewing and carcinoma of the buccal mucosa. It also became apparent that though over 50 per cent of the general population chewed betel and nut only 8·7 per cent of oral carcinoma patients were pure betel and nut chewers. Lime is always used with betel and nut and could not be, therefore, a factor of much importance. There could be no doubt in our minds that the most important single aetiological factor in carcinomas of the buccal mucosa was the habit of tobacco, betel and nut chewing in combination.

We also felt that the tobacco, betel and nut chewing by itself was not the sole factor and that dental sepsis was another. As we have already stated the lower middle class population is as large, if not larger, than the labour population and is as much addicted to the habit of tobacco, betel and nut chewing in the rural areas. Yet the incidence of oral carcinoma was nearly twice as much in labour

as in the lower middle class. The nutritional quality of the diet has very little to do with this as more often than not the labour diet is more nutritious. Labour spends virtually all its earnings on food while the lower middle class with only a slightly higher income has to expend on many subjects other than food. Both labour and lower middle class suffer from an equal degree of overall vitamin deficiency. There were as many angular stomatitis cases in the study group as in the control group and as many cases of xerosis of the conjunctiva. The only significant difference between the lower middle class and the labour lay in their personal and oral hygiene. We feel that gross dental sepsis was the main contributory factor in the higher buccal mucosal cancer incidence in labour.

Tongue

Next to the cheek, the tongue was the most important site of squamous cell carcinoma. It constituted 22 per cent of all the intra-oral carcinoma. However, the tongue carcinomas were less than half the number of cheek carcinomas. Khanolkar's (1944) figures from Bombay are the very reverse of this.

Author	Place	Cancer of cheek (%)	Cancer of tongue (%)
Khanolkar (1944)	Bombay	16	52
Cancer Institute (Madras)	Madras	51·5	22·1

Again the site distribution of the carcinomas in the tongue is very different in Bombay and Madras :

Author	Place	Cancer of tongue	
		Anterior 2/3 (%)	Posterior 1/3 (%)
Sanghvi, Rao and Khanolkar (1955)	Bombay	11·4	45·9
Cancer Institute	Madras	10	6·9

(The figures are the percentages of all oral carcinoma.)

These contrasting figures again emphasise the role of the chewing habit in the occurrence of our oral carcinomas.

Dr. Khanolkar attributes the high incidence of the carcinoma of the posterior one third of the tongue in Bombay to the combined habit of smoking bidis and chewing tobacco.

Table IV is an analysis of the possible aetiological factors in carcinoma of the tongue :

Fractional test meals were not done as they were not thought to be of significance in the analysis of the carcinoma of cheek cases.

The study of the figures given above suggests certain inferences :

The males predominate, the percentage is higher than in cheek carcinoma, the predominance being especially marked in the posterior tongue carcinomas (91·6 per cent).

There is a higher incidence in the Moslems than their general population figures would allow and higher than in carcinoma of the cheek.

The percentages in the three social strata are very different from those in cheek carcinoma. The incidence of carcinoma in the anterior two thirds of the tongue is very much higher in the lower middle class than in labour and is very

TABLE IV.—*Patients with Carcinoma of Tongue Compared with a Control Group*

Factor studied	Carcinoma tongue Group total No. 71		Control group Group No. 278
	Anterior 2/3 (%)	Posterior 1/3 (%)	(%)
1. Sex—			
Males	75	91·6	50
Females	25	8·4	50
2. Religion—			
Hindus	75·2	81·8	84·99
Christians	5·7	0	2·5
Moslems	19·1	18·2	9·93
3. Social status—			
Upper middle class	2·88	23·8	Data not
Lower middle class	60	52·4	available
Labour	37·2	23·8	
4. Environment—			
Rural	69	59	75·6
Urban	31	41	24·4
5. Diet—			
Vegetarian	17·1	33	20
Non-vegetarian	82·9	67	80
6. Habits—			
Betel, nut and tobacco chewing	80·5	33	12·5
Betel and nut chewing	16·6	29	51·8
Smoking	69·4	58·3	47
Alcohol	22·2	8·3	Data not available
7. Family history—			
Cancer in the family at any site	2·8	4	5·7
Oral cancer in the family	—	—	Not taken
8. Pre-existing illness—			
Dental sepsis	100	75	50
Syphilis	14·2	4	3·57
Tubercle	0	4	1
Diabetes	0	0	0·7
Hypertension	2·8	16·6	0·7
Chronic glossitis	28	8·3	6
Virus infection	0	0	0
Anaemia	8·3	4	1·4

low in the upper classes. This is almost a reversal of the position obtaining in carcinoma of the cheek where labour incidence was by far the highest. The social incidence of carcinoma of the posterior third of the tongue shows another very interesting change. The figures for the upper middle class leap from ·009 per cent in cheek cancers and 2·85 per cent in the anterior tongue cancers to 23·87 per cent in the posterior tongue cancers. Labour shows the lowest incidence and the lower middle classes the highest. Considering the general labour population in the country the incidence of tongue cancer in labour is very much lower than would be expected. The lower middle classes show a slightly but definitely higher incidence than their population trend permits. It is the upper classes who exhibit a remarkably high incidence in posterior tongue cancers when account is taken of the fact that they constitute only a microscopic minority of the population.

The posterior tongue cancers also show a higher urban incidence compared to the control group.

Diet seems of no significance nor does there appear to be any familial or hereditary tendency.

The betel and nut and tobacco chewing habit in the anterior tongue cancer patients is as high (80·5 per cent) as in the carcinomas of the cheek and significantly higher than in the control group, while in the posterior tongue cancer patients there is a sharp decline in the chewing figures to 33 per cent. The incidence of the tobacco smoking habit shows a sharp rise in both the anterior tongue cancers (69 per cent) and posterior tongue cancers (58 per cent) over the cheek cancer (26 per cent) and is also higher than in the control group. Pure betel and nut chewing appears to be of no significance nor the alcohol habit.

The incidence of dental sepsis in anterior tongue cancers is 100 per cent and in posterior tongue cancers 75 per cent.

There does not seem to be any other factor of significance in pre-existing local pathology.

DISCUSSION

The foregoing study, and more convincing still the histories that are daily elicited in our outpatient department, have convinced us all that the habit of prolonged combined tobacco, betel and nut chewing over a long period (15–20 years) together with the oral sepsis thus engendered is the most important single factor in the causation of oral squamous cell carcinomas. Sex is of no importance and follows only the trend of the chewing habit. Over 90 per cent of our patients and our controls suffered from some degree of clinically obvious vitamin A and vitamin B deficiency. There was no clinical evidence of vitamin C and D deficiency. Anaemia did not seem to be of much significance. The social incidence also followed only the habit trends. The maximum tobacco, betel and nut chewers were in the lower middle classes and labour. The upper classes rarely chew tobacco. Oral hygiene amongst labour is totally lacking while it is very much better in the lower middle class. This may explain the lower incidence of carcinoma of the cheek in the lower middle classes than in the labour. Another interesting fact that we noticed generally was that the variation in cheek carcinoma incidence from one region to another of our and neighbouring states followed the tobacco, betel and nut chewing habit rather closely. We would have very much liked to study more carefully this aspect of geographical pathology had our finances permitted it. Another interesting fact was that the cheek carcinomas usually occurred on the side against which the chewer held the tobacco bolus. The chronic chewer chews a compound of dried and cured tobacco leaf, powdered betel nut and betel leaves with a touch of moistened and powdered lime. He intermittently chews this material and then holds the chewed bolus against one or the other cheek in the alveolo-labial sulcus for a while and then chews it again. This process goes on continuously for a few hours and is then replaced by a fresh chew. It is virtually chain chewing. The father passes on the habit to the son and all our cancer patients have been chewers for not less than 15–20 years, commencing usually at the age of 20 when they enter the father's profession. Most chewers have the habit of holding the bolus against one particular cheek.

Tobacco, betel and nut chewing and sepsis seem to have a very definite relationship to squamous cell carcinoma of the anterior two thirds of the tongue. The relationship follows very closely that found in cheek carcinomas. One additional factor has, however, also to be considered and that is tobacco smoking.

69 per cent of anterior tongue cancer patients were smokers compared to 26 per cent of cheek cancer patients and 47 per cent in the control group. Over 80 per cent of our patients were cheroot or bidi smokers. A beedi is just a roll of specially prepared dried tobacco leaves tied with a thread. There is no enclosing paper as in a cigarette. It is about an inch and a half long and expanded at one end. It is smoked at the narrower end. It is possible that the inhaled tobacco smoke makes its own carcinogenic contribution.

Tobacco smoking appears to assume a more important role in carcinoma of the posterior third of the tongue. Tobbacco, betel and nut chewers in posterior tongue cancers were only 33 per cent compared to 85 per cent in cancers of the cheek and 80 per cent in anterior tongue cancers, but tobacco smokers were 58·3 per cent. However, the smokers in the control were 47 per cent. There is one more significant fact and that is the comparative high incidence of posterior tongue carcinomas in the upper classes of the social strata. Could this be due to the comparatively greater prevalence of smoking and heavier smoking in that opulent class? But it is reasonable to believe that a substance that is definitely carcinogenic in the oral cavity could contribute to carcinogenesis elsewhere.

The greater frequency of posterior tongue cancer in Bombay, in the urban population in Madras State and in Moslems and in males compared to the control figures also suggests the carcinogenic role of tobacco smoking. It is well known that the smoking habit is much more common in the urban population and in Moslems in Madras State than in rural areas or other communities. Women, too, very rarely smoke.

It is our conviction that adequate vitaminisation may to a certain extent act as a protective factor against the epithelial alterations that finally summate into malignancy. Avitaminosis in itself does not, however, contribute to carcinogenesis.

(Note : Only those who smoked 10 beedis a day for over 10 years were considered regular smokers.)

SUMMARY

Cancer of the mouth and pharynx constituted 48 per cent of all malignancies seen at the Cancer Institute, Madras, of which 71 per cent were of the buccal mucosa and 22 per cent of the lingual mucosa. 347 cases of squamous cell carcinoma of the mouth were analysed for possible aetiological factors. Sex, religion, environment, diet, pre-existing illness like anaemia, syphilis, tuberculosis, diabetes, hypertension and virus diseases were not of significance. There was no relationship between achlorhydria and oral cancer. The habit of tobacco and betel and nut chewing appeared most significant. The carcinogenic action of these seem to be promoted by dental sepsis.

In carcinomas of the anterior two thirds of the tongue, the same aetiological factor held good though tobacco smoking apparently played a dominant role in carcinoma of the posterior third of the tongue.

REFERENCES

KHANOLKAR, V. R.—(1958) ' Cancer ' Edited by Ronald Raven. London (Butterworths) Vol. 3, p. 272.—(1944) *Cancer Res.* **4**, 313.

SANGHVI, L. D., RAO, K. C. M. AND KHANOLKAR, V. R.—(1955) *Brit. med. J.*, **1**, 1111.

SINUS CELL HYPERPLASIA OF LYMPH NODES REGIONAL TO ADENOCARCINOMA OF THE BREAST AND COLON

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LYMPH nodes in the immediate neighborhood of a primary tumor not infrequently show hyperplasia of the cells lining their sinuses—a lesion that various authors have described as sinus catarrth (Willis, 1934), lymphoid-histiocytic follicular reticulosis (Robb-Smith, 1946; Marshall, 1956), sinus histiocytosis (Black, Kerpe, and Speer, 1953), and sinus cell hyperplasia. In humans sinus cell hyperplasia has been observed in lymph nodes regional to carcinoma of the uterine cervix (Ries, 1901; Courtois-Suffit, 1901; Vinay, 1900; Gellhorn, 1902; Kroemer, 1904; Schindler, 1906; Goldman, 1907), carcinoma of the breast (Black, Kerpe, and Speer, 1953; Black, Opler and Speer, 1954, 1955, 1956; Black and Speer, 1957; Gnirs, 1954; Berg, 1956), carcinoma of the stomach (Black, Opler and Speer, 1954, 1956), and of the liver (Fahr, 1923), as well as in teratoma of the testis (Black, Kerpe and Speer, 1953), malignant melanoma (Black, Kerpe and Speer, 1953). In animals sinus cell hyperplasia has been described in the lymph nodes of mice bearing transplanted tumors (Homburger, 1948).

The nature of the sinus cell hyperplasia is not clear and different authors have interpreted its significance differently. Some believe that the hyperplasia is an indication of host resistance, others that it has nothing to do with host resistance and still others that it develops because of secondary changes in the tumor such as necrosis and infection. In view of these differences of opinion and of the small number of studies that have been made, it seemed likely that a re-investigation of the matter might provide additional information that would help resolve some of the issues and so the present study of sinus cell hyperplasia was undertaken.

In the early years of the nineteenth century, a number of gynecologists noted that, even in the absence of metastases, enlargement of the pelvic lymph nodes sometimes accompanied carcinoma of the uterine cervix (Ries, 1901; Kroemer, 1904; Courtois-Suffit, 1901; Vinay, 1900; Schindler, 1906). These authors observed that the enlargement was sometimes, but not always, associated with "a septic, ulcerative process in the cancerous growth", and conversely that enlargement was not necessarily always present when there was ulceration of the cancer. Microscopic examination of such enlarged nodes disclosed sinus cell hyperplasia and large lymphoid follicles with conspicuous germinal centers. In addition Kroemer (1904) observed that the hyperplastic nodes remained free of cancer for long periods of time although sooner or later metastases developed. Because of this observation, he suggested that the reaction in the lymph node might act as a barrier to metastases, although he did not believe the evidence indicated that lymph nodes actually destroyed cancer cells. Kroemer's opinions

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have received both pathological and experimental support (Goldmann, 1907; Yoffey, 1932). Schindler (1906) also reported that in his experience metastases were less common in lymph nodes that showed sinus cell hyperplasia than in those that did not. Vinay (1900) and Courtois-Suffit (1901), differentiated three forms of lymphadenopathy in cancer of the uterine cervix, 1) precancerous inflammatory enlargement and fibrosis, 2) cancerous lymphadenopathy, and 3) inflammatory lymphadenopathy. Most recent workers, however, have rejected the concept of a precancerous period of preparation of lymph nodes for metastases (Willis, 1934).

Despite the early recognition by gynecologists of alterations in the lymph nodes regional to a cancer, most students of tumors have paid little attention to the changes believing they were in no way distinctive and to be ascribed to a variety of causes: to the effects of microorganisms, to absorption of extravasated blood pigments or lipids, to retention of secretions, particularly in the breast (Ewing, 1940; Willis, 1934; Symmers, 1951; Marshall, 1956), to decomposition of the primary tumor (Kitain, 1922; Geller, 1950), to metabolic products of the primary tumor (Walther, 1948) or simply to " non-specific changes " (Willis, 1934).

In 1953, Black, Kerpe and Speer made a systematic study of the regional lymph nodes in 226 patients with mammary cancer and concluded that " the lymph nodes in an appreciable percentage of patients with breast cancer show marked sinusoidal and follicular histiocytic transformation as well as amyloid-like changes. Such changes are more prominent in cases with longer survival. A direct correlation was found also between the occurrence of such changes in the nodes and the survival of individual cases. On the other hand, no relationship was observed between the lymph node appearance and the age of the patient, or the histopathologic type of the primary tumor." They estimated that in approximately 20–40 per cent of cases of breast cancer, the axillary nodes exhibit sinus cell hyperplasia of moderate or marked degree. Among 19 patients who lived less than three years after operation, 79 per cent had no significant hyperplasia whereas among 53 patients who lived five years or more, 59 per cent had sinus cell hyperplasia. Black, Kerpe and Speer suggested these changes afford an accurate basis for prediction of five-year survival and that patients with marked sinus histiocytosis might be assured of a five-year cure in almost every case regardless of axillary metastases. These authors, however, noted that, even when the axillary nodes showed no sinus hyperplasia, 30 per cent of their patients lived at least five years after operation.

Berg (1956) confirmed the findings of Black, Kerpe and Speer with regard to lymph nodes regional to breast carcinoma, but interpreted the findings differently. Berg found that " when the size of the primary cancer and the amount of axillary metastases were held constant the degree of histiocytosis was the same whether or not the patients died of carcinoma within three years after radical mastectomy. It thus appears that the observed association of low histiocytosis and poor prognosis is indirect only and dependent upon known prognostic factors, primarily the quantity of axillary metastases and the time of operation." Berg also reported wide variations in the amount of histiocytosis in nodes from a given patient.

This survey of the literature makes it clear that an appreciable percentage of lymph nodes regional to a primary carcinoma may become enlarged even in the absence of metastases and that such nodes may show dilatation of lymphatic sinuses and hyperplasia of the cells lining them. The evidence also indicates that lymph nodes with sinus cell hyperplasis at the time of operation have fewer

metastatic deposits than do nodes without hyperplasia and that statistically patients with sinus hyperplasia have a better prognosis than do patients without such hyperplasia. The meaning of these observations is, however, not agreed upon. Some observers hold that the sinus cell hyperplasia is an indication of host resistance while others doubt that the available evidence supports this idea.

In addition to sinus cell hyperplasia, some authors have also reported finding granulomatous lesions in the lymph nodes adjacent to a cancer (Wolbach, 1911 ; Nickerson, 1937 ; Nadel and Ackerman, 1950 ; Gherardi, 1950 ; Symmers, 1951 ; Black, Kerpe and Speer, 1953 ; ten Seldam, 1956 ; Wartman, 1956 ; Gorton and Linell, 1957). The granulomas consist of numerous epithelioid cells, a few multinucleated giant cells and small foci of fibrinoid necrosis. The lesions do not contain stainable micro-organisms and are found in regional but not in distant lymph nodes. The granulomas have been variously described as tuberculoid or sarcoid, but there is no evidence that they are due either to tubercle bacilli or are a part of generalized Broeck's sarcoidosis, and therefore the term " granuloma " seems preferable. The factors that cause the granulomas to form are unknown but the following have been considered : products of neoplastic growth or breakdown probably of lipid nature (Symmers, 1951 ; Refvern, 1954 ; ten Seldam, 1956 ; Gorton and Linnell, 1957), radiation of the primary tumor (Larsson, 1949 ; Gorton and Linnell, 1957), infection of the primary tumor and hypersensitivity (ten Seldam, 1956).

Deposits of hyaline or amyloid-like material in the sinuses and pulp of the lymph nodes have also been described and are thought to be in some way related to sinus cell hyperplasia or granuloma formation (Symmers, 1951 ; Black, Kerpe and Speer, 1953).

MATERIAL AND METHODS

All cases of adenocarcinoma of the female breast recorded in the files of the Bland-Sutton Institute of Pathology, Middlesex Hospital, London, for the year 1951 were reviewed. The patients had been treated by radical mastectomy and in most cases histologic sections of the primary tumor and axillary lymph nodes stained with hematoxylin and eosin were available for microscopic study. In addition, follow-up studies had been made so that it was known whether the patients were (1) living and free of cancer, (2) living but with clinical evidences of cancer, (3) dead of cancer, or (4) dead of unrelated cause. Althogether there were 103 cases in the files and of these 66 had histologic sections of both the primary tumor and axillary lymph nodes (404 nodes) as well as adequate follow-up data. These 66 cases were analyzed with respect to (1) sinus cell hyperplasia in the lymph nodes, (2) metastases, (3) histologic grade of the primary tumor, (4) length of survival after operation, (5) inflammation and necrosis of the primary tumor, (6) follicular hyperplasia, (7) granuloma formation, and (8) hyaline deposits in the lymph nodes.

A similar analysis was made of patients with primary adenocarcinoma of the colon operated on at Middlesex Hospital during the year 1956. This particular year was chosen because a special study of carcinoma of the colon had been started that year and all lymph nodes removed by the surgeon had been prepared for microscopic examination. There were twenty-four cases with 303 lymph nodes.

The degree of sinus cell hyperplasia was graded empirically as absent, slight, moderate or marked, but for the purposes of the tables only nodes with moderate

or marked changes were considered as showing sinus cell hyperplasia, whereas nodes with slight changes were classified with those showing none at all. Not every node in a given case showed sinus cell hyperplasia and at times the hyperplasis in individual nodes was focal rather than diffuse. Such variation, however, is not recorded in the tables which show simply whether or not hyperplasia was present in any of the nodes.

The primary carcinomas in both breast and colon were graded histologically according to the method of Patey and Scarff (1928, 1929) and Scarff (1952) which is based on the recognition of two factors—anaplasia and rapid growth. Anaplasia is recognized by absence of tubule formation ; and rapid growth by irregularity of nuclei, hyperchromatism and mitosis. By use of this method the tumors were sorted as follows : Grade 1 carcinomas showed only slight anaplasia and evidences of slow growth, Grade 2 carcinomas showed moderate anaplasia and evidences of moderately rapid growth and Grade 3 tumors anaplasia and rapid growth.

RESULTS

Microscopic examination of the lymph nodes showed that 50 per cent of cases of breast carcinoma and 33 per cent of cases of colon carcinoma had sinus cell hyperplasia (Table I). The changes consisted of dilatation of the lymphatic sinuses, often most pronounced in the medullary sinuses, and hyperplasia, proliferation and desquamation of the cells lining the sinuses. These cells had abundant, faintly eosinophilic cytoplasm which often appeared amoeboid, stretching out and branching laterally at the poles of the cells. Some cells were continuous with similar ones on the wall of the sinus and formed a syncytium. The cells stained positively with the Weil-Davenport method, and were thought to be littoral cells (the metalophil cells of Marshall (1956) or the reticular cells of Maximow and Bloom (1942). These findings confirm the observations of others.

Correlation of Sinus Cell Hyperplasia with Metastases, Length of Survival and Histologic Grade of the Primary Tumor.

Analysis of the cases according to whether or not tumor deposits were discovered in the nodes removed at operation showed that sinus cell hyperplasia occurred more frequently in patients free of metastases than in those with metastases (Table I). In breast carcinoma only 36 per cent of patients with sinus cell hyperplasia had metastases in contrast to 74 per cent of those without it. Similarly in colon carcinoma only 12 per cent of patients with sinus cell hyperplasia in the

TABLE I.—*General Incidence of Sinus Cell Hyperplasia and Correlation with Lymph Node Metastases and Survival*

Lymph nodes	Per cent of total cases	Per cent of cases with positive nodes	Per cent of cases alive 7 years
	Breast Carcinoma (66 cases)		
With hyperplasia	50	36	64
Without hyperplasia	50	74	39
	Colon Carcinoma (24 cases)		
With hyperplasia	33	12	—
Without hyperplasia	67	50	—

regional lymph nodes had metastases whereas 50 per cent of patients without sinus cell hyperplasia had metastases. It should be noted, however, that although the same relative differences were apparent, the absolute incidence was less in patients with colon carcinoma than in those with breast carcinoma.

Table I shows a similar difference with respect to survival in patients with breast carcinoma: 64 per cent of patients who had sinus cell hyperplasia at the time of operation were alive and apparently free of cancer seven years later, while only 39 per cent of those without sinus cell hyperplasis lived so long. Survival data for colon carcinoma were not available.

A positive correlation was also apparent for the histologic grade of the tumor and the occurrence of sinus cell hyperplasia for 64 per cent of Grade 1 carcinomas of the breast showed sinus cell hyperplasia but only 34 per cent of Grade 3 tumors (Table II). In carcinoma of the colon, 40 per cent of Grade 1 tumors showed sinus cell hyperplasis and none of Grade 3 tumors.

TABLE II.—*Correlation of Histologic Grade of the Primary Tumor with Sinus Cell Hyperplasia of Regional Lymph Nodes and Survival of Patients*

	Breast Carcinoma			*Colon Carcinoma*	
	Per cent total cases	Per cent with hyperplasia	Per cent alive 7 years	Per cent total cases	Per cent total with hyperplasia
Total	—	50	53	—	33
Grade 1	21	64	57	42	40
Grade 2	43	55	59	29	57
Grade 3	35	34	43	29	0

Correlation of Sinus Cell Hyperplasia with Inflammation and Necrosis of the Primary Tumor

Repeatedly it has been suggested that inflammation and necrosis of the primary tumor cause sinus cell hyperplasia. The data bearing on this point from the present series of cases of breast and colon cancer have been set out in Tables III and IV.

TABLE III.—*Correlation of Inflammation or Necrosis of Primary Tumor with Sinus Cell Hyperplasia and Follicular Hyperplasia of Regional Lymph Nodes*

	Breast Carcinoma			*Colon Carcinoma*		
	Per cent total cases	Per cent with sinus hyperplasia	Per cent with follicular hyperplasia	Per cent total cases	Per cent with sinus hyperplasia	Per cent with follicular hyperplasia
With inflammation or necrosis	37	56	13	71	29	27
Without inflammation or necrosis	63	50	20	29	43	35

Table III gives the percentage of cases with inflammation or necrosis of the primary tumor which also had hyperplasia. Although 37 per cent of breast carcinomas and 71 per cent of colon carcinomas showed either inflammation or necrosis, only a half of the breast and a quarter of the colon tumors had sinus cell hyperplasia in regional lymph nodes. Thus inflammation and necrosis were

TABLE IV.—*Correlation of Nodal Metastases with Follicular Hyperplasia, Granulomas and Hyaline Deposits*

	Per cent total cases	Per cent with follicular hyperplasia	Per cent with granulomas	Per cent with hyaline
		Breast Carcinoma		
All cases	—	4	5	26
Cases with negative nodes	44	6	9	36
Cases with positive nodes	56	2	2	17
		Colon Carcinoma		
All cases	—	29	8	3
Cases with negative nodes	62	37	13	7
Cases with positive nodes	38	33	0	0

not always associated with sinus cell hyperplasia and it would seem that other factors must have operated in these cases. Careful microscopic examination of the lymph nodes from the cases with inflamed or necrotic primary tumors also failed to reveal the expected evidences of inflammatory lymph adenopathy such as follicular hyperplasia (Table III), phagocytosis, lipid deposits, abscesses or accumulations of plasma cells.

Follicular Hyperplasia, Granulomas and Hyaline

A small proportion of cases of both breast and colon carcinoma showed in regional lymph nodes an increased number of large follicles with prominent germinal centers, granulomas of sarcoid type, or hyaline deposits (Table IV). Follicular hyperplasia occurred more frequently in carcinoma of the colon and hyaline deposition in carcinoma of the breast. In both types of cancer, granuloma and hyaline were more frequent in nodes that did not contain metastases than in those that did.

DISCUSSION

In this study one half the patients with breast carcinoma and one third of those with colon carcinoma had sinus cell hyperplasia of regional lymph nodes at the time of operation and analysis of the cases indicated that patients with sinus cell hyperplasia had developed fewer metastases in regional lymph nodes than had patients without such changes. Further, in patients with both metastases and sinus cell hyperplasia, the hyperplasia occurred chiefly in the nodes in which no tumor was discovered. These findings confirm those of other workers (Black, Kerpe and Speer, 1953; Black and Speer, 1958; Berg, 1956). Comparison of the histologic grade of the primary tumor and the prevalence of sinus cell hyperplasia in the regional nodes showed that it was present in 64 per cent of Grade 1 tumours of the breast but in only 34 per cent of Grade 3 tumors. In colon carcinoma a similar difference was obvious for 40 per cent of Grade 1 tumors had sinus hyperplasia, but none of Grade 3 tumors. Patients with nodal sinus hyperplasia also had a better chance of surviving for seven years after operation than did patients without sinus cell hyperplasia.

Although these findings suggest that host resistance to the tumor may have occurred, we must not overlook the possibility that the changes in the lymph nodes represent a reaction not to the tumor but to something else. One possibility

s that necrosis and inflammation in the primary tumor may have caused the eaction. This possibility was investigated but the data on it are not clear-cut erhaps because of the nature of the material which was studied. Thus only the ritten descriptions of the tumors and the ordinary microscopic sections were vailable for determining the presence and severity of the necrosis and inflammaion and it is possible that they may have been overlooked in the gross examinaion of the specimens or that the histologic sections did not include the areas of nflammation and necrosis. Nevertheless some information on the point can be btained by analysis of the cases in which inflammation and necrosis were disovered. For example, in breast cancer only about half the cases with necrosis nd inflammation of the primary tumor showed sinus cell hyperplasia and in colon arcinoma somewhat less than a third (Table II). Necrosis and inflammation ere commoner in colon than in breast carcinoma—71 as contrasted with 37 per ent—but the incidence of hyperplasia was just the reverse—29 per cent of colon nd 56 per cent of breast carcinomas. The microscopic appearance of lymph odes with sinus cell hyperplasia was also different from that commonly seen in nflammatory lymphadenopathy, since follicular hyperplasia was not frequent and there were no abscesses, phagocytes or collections of plasma cells. It might lso be expected that carcinoma of the colon, being more frequently infected than carcinoma of the breast, would show more sinus cell hyperplasia in regional lymph nodes but this was not the case. These findings suggest that although necrosis and inflammation may be one cause of sinus cell hyperplasia they are probably not the only one.

Other possible causes of sinus cell hyperplasia, such as retention of secretions could not be studied in the present material. It might be pointed out, however, that although retention of secretions may be a possibility in breast carcinoma, it does not seem to be a likely cause in carcinoma of the colon, stomach or uterine cervix where sinus cell hyperplasia also occurs.

The significance of the findings of granulomas and deposits of hyaline in some of the lymph nodes is unclear. The data in Table V, which are too few to be statistically significant, suggest that granulomas and hyaline were more common in nodes showing sinus cell hyperplasia than in those without it as well as in nodes that were free of secondary carcinoma (Table V). There was, however, no marked

TABLE V.—*Correlation of Sinus Cell Hyperplasia with Occurrence of Follicular Hyperplasia, Granulomas and Hyaline in Regional Lymph Nodes*

	Breast			*Colon*		
	Per cent with follicular hyperplasia	Per cent with granulomas	Per cent with hyaline deposits	Per cent with follicular hyperplasia	Per cent with granulomas	Per cent with hyaline deposits
All cases	5	8	35	29	8	4
Sinus cell hyperplasia	5	14	46	33	11	0
No sinus cell hyperplasia	5	3	22	27	7	7
Negative nodes	6	9	36	27	13	7
Positive nodes	2	2	17	33	0	0
Inflammation and necrosis	13	14	39	27	6	6
No inflammation and necrosis	20	4	33	35	14	0

increase in the number of granulomas formed or the amount of hyaline deposited when the primary tumor was necrotic or inflamed.

The results of this study, while agreeing in the main with those of Black and his colleagues, nevertheless differ in certain respects which may now be mentioned. Follicular hyperplasia did not occur as frequently in the cases in the present series as in Black's and there was a positive correlation between the histological grade of the primary carcinoma and the incidence of sinus cell hyperplasia. Black could not show such a correlation. The reasons for these differences in observations are not apparent but in the case of histologic grading, different methods of evaluation may have been used. Black and his colleagues suggested that the presence of sinus cell hyperplasia assured a patient of a five year survival. Our data for a seven year survival period do not show this although, in general, the presence of sinus cell hyperplasia seemed to indicate a favorable prognosis. The correlation, however, was not sufficiently good to permit prediction of life expectancy in individual cases. The data of Black and his colleagues also suggest that follicular hyperplasia, granuloma formation and hyaline deposition in regional lymph nodes are related to sinus cell hyperplasia. Our data, on the other hand, do not suggest such a relation.

SUMMARY

The occurrence of hyperplasia of the cells lining the sinuses of lymph nodes regional to a primary carcinoma has been studied in 66 patients with breast carcinoma and 24 patients with colon carcinoma. The data have been analyzed with respect to metastases in the regional lymph nodes, the histologic grade of the primary tumor, the length of survival of the patients after operation and the amount of inflammation and necrosis in the primary tumor. The data appear to support the following conclusions:

1. Sinus cell hyperplasia of regional lymph nodes occurred in a considerable proportion of patients with carcinoma—a half of those with breast cancer and a third of those with colon cancer.

2. Patients without metastases in the nodes showed sinus cell hyperplasia twice as frequently as patients with metastases.

3. Sinus cell hyperplasia correlated with the histologic grade of the primary tumor—the higher the grade of malignancy the less the sinus cell hyperplasia.

4. These correlations applied to both breast and colon carcinoma, but were more striking in the former.

5. Sixty-four per cent of patients with breast carcinoma who had sinus cell hyperplasia were alive seven years after operation, whereas only 39 per cent of patients without sinus cell hyperplasia lived that long. A similar analysis of patients with colon cancer was not done because they had not yet been followed for more than a year.

6. Inflammation and necrosis of the primary tumor were found in 37 per cent of breast carcinomas and 71 per cent of colon carcinomas. The evidence, however, suggests that factors other than inflammation and necrosis may cause the sinus cell hyperplasia.

7. These findings together with similar observations reported by other workers suggest that sinus cell hyperplasia may be an indication of host resistance to a primary carcinoma rather than a non-specific alteration.

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REFERENCES

BERG, J. W.—(1956) *Cancer*, **9**, 935.
BLACK, M. M., KERPE, S. AND SPEER, F. D.—(1953) *Amer. J. Path.*, **29**, 505.
Idem, OPLER, S. R. AND SPEER, F. D.—(1954) *Surg. Gynec. Obstet.*, **98**, 725.—(1955) *ibid.*, **100**, 543.—(1956) *Ibid.*, **102**, 599.
Idem AND SPEER, F. D.—(1957) 'Human Cancer.' Chicago (The Year Book Publishers), p. 73.—(1958) *Surg. Gynec. Obstet.*, **106**, 163.
COURTOIS-SUFFIT, M. (1901) *Bull. méd., Paris*, **15**, 445.
EWING, J.—(1940) 'Neoplastic Diseases'. Philadelphia. (Saunders).
FAHR, T.—(1923) *Virchows Arch.*, **247**, 66.
GELLER, F. C.—(1950) *Arch. Geschwulstforch.*, **2**, 171.
GELLHORN, G.—(1902) *Amer. Gynec.*, **1**, 476.
GHERARDI, G. J.—(1950) *Arch. Path.*, **49**, 163.
GNIRS, L.—(1954) *Z. Krebsforsch.*, **60**, 94.
GOLDMANN, E.—(1907) *Proc. Roy. Soc. Med.*, **1**, 14, Surg. Sect.
GORTON, G. AND LINELL, F.—(1957) *Acta radiol., Stockh.*, **47**, 381.
HOMBURGER, F.—(1948) *Science*, **107**, 648.
KITAIN, H.—(1922) *Virchows Arch.*, **238**, 289.
KROEMER, P.—(1904) *Arch. Gynaek.*, **73**, 57.
LARSSON, L. G.—(1949) *Acta radiol., Stockh.*, **31**, 17.
MARSHALL, A. H. E.—(1956) 'An Outline of the Cytology and Pathology of the Reticular Tissue'. London (Oliver and Boyd), p 120.
MAXIMOW, A. A. AND BLOOM, W.—(1942) 'A Textbook of Histology'. Philadelphia (W. B. Saunders Co.), p. 271.
NADEL, E. M. AND ACKERMAN, L. V.—(1950) *Amer. J. clin. Path.*, **20**, 952.
NICKERSON, D. A.—(1937) *Arch. Path.*, **24**, 19.
PATEY, D. H. AND SCARFF, R. W.—(1928) *Lancet*, i, 801.—(1929) *Ibid.*, ii, p. 492.
REFVERN, O.—(1954) *Acta med. scand.*, Suppl., 294.
RIES, E.—(1901) *Amer. J. Obstet., Dis. Wom.*, **44**, 29.
ROBB-SMITH, A. H. T.—(1946) *Spec. Rep. Ser. med. Res. Coun. Lond.*, No. 259.
SCARFF, R. W.—(1952) *Arch. Middx Hosp.*, **2**, 174.
SCHINDLER, R.—(1906) *Mschr. Geburtsh. Gynäk*, **23**, 502.
TEN SELDAM, R. E. J.—(1956) *Med. J. Aust.*, **1**, 916.
SYMMERS, W. St. C.—(1951) *Amer. J. Path.*, **27**, 493.
VINAY, C.—(1900) *Lyon. méd.*, **95**, 289.
WALTHER, H. E.—(1948) 'Krebsmetastasen'. Basel (Benno Schwabe & Co.), p. 86.
WARTMAN, W. B.—(1956) 'Year Book of Pathology and Clinical Pathology'. Chicago (Year Book Publishers), p. 74.
WILLIS, R. A.—(1934) 'The Spread of Tumors in the Human Body'. London (J. & A. Churchill), p. 31.
WELBACH, S. B.—(1911) *J. med. Res.*, **24**, 253.
YOFFEY, J. M.—(1932) *Brit. med. J.*, ii, 1052.

FLUORESCENCE MICROSCOPY IN EXFOLIATIVE CYTOLOGY

An Evaluation of its Application to Cancer Screening

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The concentration of fluorescent dyes (fluorochromes) by neoplasms has been utilized for in-vivo localization (Cramer and Brilmayer, 1952; Hubbard and Moore, 1949), and for quantitative cytochemical analyses in microscopic sections or smears (Cunningham, Griffin and Luck, 1950; Davidson, Leslie and White, 1951; Mellors, Glassman and Papanicolaou, 1952; Moberger, 1954). Fluorescent chemical groups which may be excited by ultra-violet or blue-violet irradiation include the xanthines, acridines, thiazoles, azo-dyes and some alkaloids (Hicks and Matthaei, 1955). The most remarkable feature of fluorochromes is their ability to permit color visualization in dilutions at which the daylight color of the strongest dyes is imperceptible (Metcalf and Patton, 1944). Frozen sections, paraffin sections or smears of fresh or fixed tissues may be used. The staining procedures are simple and rapid, and the fluorescence may be accentuated by treatment with weak acids or alcohols (Peacocke and Skerrett, 1956; Vinegar, 1956). Many fluorochromes possess metachromasia which permits sharp differential staining without the use of counterstains, the color differences being dependent upon variation in ion concentration, selective absorption and natural fluorescence.

Fluorescence microscopy (FM) has been applied successfully to qualitative and quantitative analyses of the nucleoproteins, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in normal, regenerating, hyperplastic and neoplastic cells (Hicks and Matthaei, 1955; Krieg, 1953; Price and Laird, 1950; Vinegar, 1956). Since non-viable cancer cells retain their power to combine with fluorescent dyes (Moberger, 1954) the opportunities for the use of FM in exfoliative cytology were soon recognized. Friedman (1950) reported on its use in the detection of malignant cells in vaginal smears. He experimented with a variety of stains and recommended a combination of berberine sulfate, acid fuchsin and acridine yellow. Mellors *et al.* (1952) demonstrated a linear relation between the fluorescence of berberine sulfate and the chromosome number, nucleic acid content and nuclear extinction, resulting from the binding of the basic dye with the acidic constituents of chromatin and cytoplasmic nucleoproteins. Bertalanffy and Bickis (1956) and Bertalanffy, Masin and Masin (1956, 1958) using acridine orange (AO) concluded that FM was a practical method for the screening of vaginal smears. They demonstrated the specific staining of DNA and RNA by AO, using as controls histochemical procedures and specific enzymes.

This study was undertaken to determine the usefulness of AO-FM as a screening procedure in routine diagnostic exfoliative cytology.

MATERIALS AND METHOD

Smears of 1,295 specimens of sputum or bronchial aspirates, serous cavity fluids, urines, gastric washings, and smears from irradiated oral carcinomas were fixed in ether-alcohol for at least 5 minutes and stained using a modification of the technique of Bertalanffy and Bickis (1956).* The stained preparations were examined in a semi-darkened room, using the Zeiss fluorescence apparatus with blue-violet radiation.† The smears were scanned at a magnification of 125 without the use of a mechanical stage. The mean examination time was 3.8 minutes for an average of 3 smears per specimen. The results were recorded as positive or negative.

At the completion of the AO-FM examination, the coverglasses were removed, the smears immersed in 50 per cent ethyl-alcohol for several minutes, replaced in ether-alcohol for 15 minutes and then restained by the Papanicolaou technique. The mean examination time was 8 minutes by the Papanicolaou method.

RESULTS

The staining procedure was simple and rapid, and morphological details were excellent. The fluorescent features of the various normal and malignant cells have been so well described and illustrated by Bertalanffy and his associates (1956 and 1958), that they will not be presented in detail here.

Even at low magnification malignant cells were detected quickly because of their brilliant fluorescence against a dark background in which normal squamous cells were inconspicuous, the dull green fluorescence of nuclei and cytoplasm rendering them almost invisible. The complete lack of fluorescence of erythrocytes was helpful, especially in the examination of bloody smears.

The nuclei of malignant cells were green (low DNA) to yellow or yellow-white (high DNA), while cytoplasm and nucleoli were intense orange or red (high RNA), except for RNA deficient malignant cells which had green cytoplasmic fluorescence.

Normal gastric and respiratory columnar cells, some atypical or metaplastic squamous cells, regenerating transitional cells, histiocytes and mesothelial cells had orange cytoplasm and yellow nuclei, as did the malignant cells. Despite the tinctorial similarity, the fluorescence of the benign cells was not as bright as *typical* malignant cells, but occasionally exceeded that of poorly fluorescing cancer cells. In these instances morphological features had to be relied upon for differentiation.

Leukocytes were troublesome occasionally, since they exhibited bright yellow fluorescence of nuclei. Usually this was of no consequence because of the small size of the leukocytes, but when present in tight clusters or large masses they sometimes camouflaged malignant cells concealed therein. Nevertheless the bright orange cytoplasm permited the recognition of cancer cells in some clusters

* Hydrate through alcohols (80 per cent, 70 per cent, 50 per cent) to distilled water. Rinse in 1 per cent acetic acid (5 dips). Wash in distilled water. Stain in acridine orange (National Aniline), 1 : 40,000 dilution in phosphate buffer at pH 6·0 (12·9 ml. of M·15 Na_2HPO_4 and 87·1 ml. of M·15 NaH_2PO_4). Destain in phosphate buffer, 1–1½ minutes. Differentiate in 1M $CaCl_2$, 1–1½ minutes. Mount in phosphate buffer.

† Maximum pressure mercury vapor lamp with filters UG2 and UG5 (5000–8000 Å), two barrier filters OG5.

of leukocytes which completely obscured them in the Papanicolaou preparations.

In general the AO-FM findings corresponded with those of the controls. Nuclei which were hyperchromatic in the Papanicolaou preparations fluoresced brightly. The brilliant orange fluorescence of nucleoli was striking, and readily differentiated nucleoli from clumped chromatin which was green or yellow. Multiple small nucleoli which were not discernible by the Papanicolaou stain were revealed beautifully. The most significant difference between the interpretation of malignancy by the two techniques was in the relative importance of the appearance of the cytoplasm. Cytoplasmic features are of relatively little importance in the diagnosis of malignancy by the Papanicolaou technique, which depends mainly on nuclear characteristics. The characteristics of the cytoplasm are utilized chiefly for determination of cell type. On the other hand, the flaming orange-red fluorescence of the cytoplasm of malignant cells was the most striking feature of AO-FM. The brightest cytoplasmic fluorescence was seen in cells which had deeply basophilic cytoplasm by the Papanicolaou stain.

Vacuoles neither fluoresce, nor absorb the dyes in the Papanicolaou stain, and therefore were equally well demonstrated by both techniques because of the contrast with the adjacent stained cytoplasm.

Acridine orange did not demonstrate keratin and the squamous nature of malignant cells had to be identified by morphological features. Although the differentiation of cornified and non-cornified cells is of secondary importance in exfoliative cytologic cancer diagnosis, the bright orange appearance of cornified cells by the Papanicolaou stain does facilitate their detection during scanning. The failure of some cornified malignant squamous cells to fluoresce more brightly than their normal prototypes was the cause of most of the failures of detection by AO-FM. Although fluorescence usually decreased with cornification, there was no uniform correlation between the degree of keratinization and the brightness or the color of the fluorescence.

Maturation of malignant cells was manifested by decreased fluorescence. The intensity of fluorescence was not greatly affected by autolysis, and cells which appeared smudged or poorly stained in the controls often were brightly fluorescent, indicating stability of the nucleic acid binding capacity.

The comparative sensitivity of AO-FM is indicated in Table I. Malignant cells were recognized in 191 of 231 specimens (83 per cent) which were positive by the Papanicolaou method.

TABLE I.—*Comparative Sensitivity of Acridine Orange Fluorescence Microscopy.*

Type of specimen	Numbers of specimens examined	Positive by Papanicolaou method	Positive by fluorescence microscopy	Sensitivity of fluorescence microscopy (per cent)
Sputum	630	71	62	87
Oral smears	402	128	102	80
Urines	179	19	16	84
Serous cavity fluids	50	8	8	100
Gastric and esophageal washings	34	5	3	50
Total	1295	231	191	83

False positive AO-FM—37 (16.2 per cent).

The lack of increased fluorescence of some malignant cells accounted for most of the false-negative results. Most positive specimens contained some malignant cells which did have the characteristic fluorescence, but when the cancer cell population of a smear was very sparse and fluoresced poorly, only prolonged search revealed the malignant cells. Since these cells had to be identified by morphological features, they were less conspicuous than they were in the controls.

The smears from oral squamous cell carcinomas during x-ray therapy were of special interest and will be described in detail elsewhere. There was often a reduction in the fluorescence of the irradiated malignant cells during the later phases of treatment. The nuclear fluorescence usually decreased more rapidly than did that of the cytoplasm or nucleoli.

There were 37 false-positives by AO-FM. This may seem excessive, but results were interpreted as positive or negative without the use of a suspicious category into which most of the false-positives would have been classified. In addition before the authors became familiar with the variable fluorescence of atypical benign cells, such as hyperplastic mesothelial, squamous or transitional cells and atypical histiocytes, such cells were misinterpreted occasionally.

DISCUSSION

The application of acridine orange-fluorescence microscopy to exfoliative cytologic screening for cancer cells is based upon the high protein synthesis of cancerous tissue, since the fluorescence of malignant cells appears to be proportional to their DNA and RNA moities. Although most malignant cells have more nucleic acids than their normal prototypes (Mellors, Glassman and Papanicolaou, 1952 ; Moberger, 1954), some cancer cells have no significant increase in DNA (Cunningham, Griffin and Luck, 1950 ; Davidson, Leslie and White, 1951) and decreased RNA has been found in less virulent malignant cells (Caspersson and Santesson, 1942) or in mouse ascites tumors which have been stored at low temperatures (Klein, Kurnick and Klein, 1950). Thus it is not surprising that some exfoliated malignant cells failed to show sufficient fluorescence to permit their rapid recognition by AO-FM scanning. Most of the AO-FM failures in this study were due to lack of fluorescence of well differentiated squamous carcinoma cells. Since most exfoliated cells have reached their fullest maturation prior to desquamation, it is remarkable that such a high proportion still exhibits such brilliant fluorescence.

The advantages of fluorescence microscopy in exfoliative cytology are :

(1) The staining technique is simple, rapid and inexpensive.

(2) Smears which are scanned for less than 5 minutes per *set* of smears will detect the majority of specimens containing malignant cells.

(3) Most malignant cells can be recognized at a glance, and their morphologic features are distinct.

(4) Unsatisfactory specimens are recognized readily, thereby eliminating the need for the preparation of permanent preparations.

(5) The same smears may be restained by standard techniques.

The disadvantages are :

(1) Less sensitivity for most types of specimens. It was not as reliable as the Papanicolaou method, with the possible exception of serous cavity fluids.

(2) Additional equipment is required.

(3) The smears are not permanent.

(4) A semi-darkened room is necessary.

(5) Equally good results have been reported by the rapid scanning of Papanicolaou stained smears (Simon and Ricci, 1957). (In this study no attempt was made to restrict the time of examination of controls.)

(6) Eyestrain may be somewhat greater.

CONCLUSIONS

1. Twelve hundred and ninety-five exfoliative cytologic specimens of sputum, bronchial secretions, serous cavity fluids, oral smears, urine and gastric and esophageal washings were examined by blue-violet fluorescence microscopy after staining by acridine orange. The same smears restained by the Papanicolaou technique provided the controls.

2. The mean examination time per specimen (average of 3 smears per specimen) was 3.8 minutes by the acridine orange-fluorescence technique and 8 minutes by the Papanicolaou method.

3. Two hundred and thirty-one specimens were positive by the Papanicolaou method. Of these one hundred and ninety-one (83 per cent) were detected by fluorescence microscopy.

4. The diagnosis of malignancy by the Papanicolaou technique is dependent chiefly upon nuclear changes; in fluorescence microscopy the fluorescence of the cytoplasm is of equal or greater diagnostic value.

5. Not all malignant cells exhibited increased fluorescence. This was especially true of some well differentiated malignant squamous cells.

6. The advantages and disadvantages of fluorescence microscopy in exfoliative cytology have been discussed.

REFERENCES

VON BERTALANFFY, L. AND BICKIS, I.—(1956) *J. Histochem.*, **4**, 481.
Idem, MASIN, F. AND MASIN, M.—(1956) *Science*, **124**, 1024.
Idem, MASIN, M. AND MASIN F.—(1958) *Cancer*, **11**, 873.
CASPERSSON, T. AND SANTESSON, L.—(1942) *Acta radiol.* Suppl., **46**, 5.
CRAMER, H. AND BRILMAYER, C.—(1952) *Münch. med. Wschr.*, **94**, 1641.
CUNNINGHAM, L., GRIFFIN, A. C. AND LUCK, J. M.—(1950) *J. gen. Physiol.*, **34**, 59.
DAVIDSON, J. N., LESLIE, I. AND WHITE, J. C.—(1951) *J. Path. Bact.*, **63**, 471.
FRIEDMAN, H. P., JR.—(1950) *Amer. J. Obstet. Gynec.*, **59**, 852.
HICKS, J. D. AND MATTHAEI, E.—(1955) *J. Path. Bact.*, **70**, 1.
HUBBARD, T. B., JR. AND MOORE, G. E.—(1949) *J. nat. Cancer Inst.*, **10**, 303.
KLEIN, E., KURNICK, N. B. AND KLEIN, G.—(1950) *Exp. Cell. Res*, **1**, 127.
KREIG, A.—(1953) *Klin. Wschr.*, **31**, 350.
MELLORS, R. C., GLASSMAN, A. AND PAPANICOLAOU, G. N.—(1952) *Cancer*, **5**, 458.
METCALF, R. L. AND PATTON, R. L.—(1944) *Stain Tech.*, **19**, 11.
MOBERGER, G.—(1954) *Acta radiol.* Suppl., **112**, 1.
PEACOCKE, A. R. AND SKERRETT, J. N. H.—(1956) *Trans. Faraday Soc.*, **52**, 261.
PRICE, J. M. AND LAIRD, A. K.—(1950) *Cancer Res.*, **10**, 650.
SIMON, T. R. AND RICCI, A.—(1957) *Trans. Intersoc. Cytology Coun.*, **5**, 121.
VINEGAR, R.—(1956) *Cancer Res.*, **16**, 900.

CALCIFIED ATHEROSCLEROSIS AND CANCER

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THIS paper is a continuation of earlier studies in which the relationship of calcified atheroma to cancer was examined (Elkeles, 1949, 1950, 1953, 1956, 1957). It was found that calcified atherosclerosis of the abdominal aorta is significantly less common in carcinoma than in control cases of the same age groups. From these results it was concluded that individuals with noticeable calcified atherosclerosis are relatively immune to cancer.

Radiography of the abdominal aorta was used to estimate the presence or absence of calcified atheroma in patients aged more than 50. This method, in contrast to autopsy, permits the examination of a large number of unselected control cases. Moreover, calcium deposits in the aorta can be detected more easily than by macroscopic inspection and palpation of the arteries.

So far 622 carcinoma cases and 1480 controls over the age of 50 have been examined by the radiographic method. The results are shown in Fig. 1.

These results refer to carcinomata irrespective of the site of the tumour. They show a significantly lower incidence of calcified atherosclerosis in cancer than in the control cases. The incidence of calcification in the abdominal aorta in the control series rises from 19 per cent in the 50–60 age group to 65 per cent in the 71–80 age group. In the carcinoma series it rises from 10 per cent in the 50–60 age group to 32 per cent in the 71–80 age group. Thus in all three age groups the incidence of calcification of the abdominal aorta is almost twice as high in the controls as in the carcinoma cases. The same relationship was found regarding the severity of the calcified lesions.

These observations point to the possibility of a fundamental difference in the biochemical system of the two groups.

In a previous paper (Elkeles, 1956) I pointed out that the incidence of calcified atheroma is particularly low in carcinoma of the sex glands, colon and stomach. Bronchial carcinoma is one of the exceptions in which I found as least as much calcified atherosclerosis as in the control cases.

In recent years the cancer-atherosclerosis relationship has received much attention by pathologists from various countries. According to Grosse (1958) this problem has been investigated by 15 authors in over 36,000 post-mortem examinations, 11,000 of which were cancer cases. All these authors agree that the incidence and the severity of atherosclerotic lesions are significantly less pronounced in cancer than in control cases of the corresponding age groups. Since the results of my studies have been confirmed by post-mortem examinations, it is justified to assume that the negative atherosclerosis-cancer relationship is of significance for the cancer problem.

The two methods differ in that the post-mortem examinations record the incidence and severity of atherosclerosis, whereas the radiographic method determines the absence or the various degrees of calcification in the abdominal aorta.

The calcium content of aortas increases with advancing years. Already after the maturation of the skeletal system calcium salts are deposited in the media. This process is probably related to changes in the elastic tissue. The old theory that these changes in the media are the primary cause for the development of atherosclerosis has been re-instated in recent years. Blumenthal, Lansing

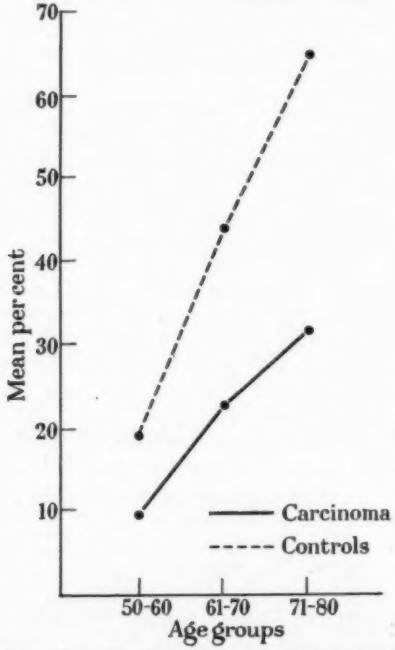

FIG. 1.—Percentage of calcification of the abdominal aorta in 622 carcinoma and in 1480 control cases over 50 years of age.

and Wheeler (1944) found a close relationship between loss of elasticity and the deposition of calcium salts in the media of the aorta and believed that these changes precede and cause the development of atheroma in the intima.

The presence or absence of "calcified" atherosclerosis in advancing years may possibly serve as an indicator for the degree of the affinity of tissue lipoids and proteins to calcium salts. The hypothesis is therefore advanced that a biochemical system showing affinity to calcium salts leads to calcified atherosclerosis and to relative immunity to cancer, a biochemical system showing more or less lack of affinity to calcium salts, is predisposing to cancer formation.

The significance of calcium in malignant tumours has been studied in many laboratories. The earliest evidence that cancer tissue contained a low amount of calcium salts was contributed by Beebe (1904–5). He found in 8 human

tumours that calcium decreased and potassium increased in proportion to the degree of malignancy. His findings were confirmed by Clowes and Frisbie (1905), who examined 100 specimens of the Jensen mouse adeno carcinoma. Eggers (1932) stated that the restraining action of calcium salts on cancerous growth appeared firmly established.

In recent years the absence or low calcium content in cancer tissue has again been emphasized by various authors. Coman (1944), McCutcheon, Coman and Moore (1948), Brunschwig, Dunham and Nichols (1946), Dunham, Nichols and Brunschwig (1946), De Long, Coman and Zeidman (1950) found the calcium contents in 12 human intestinal cancers markedly decreased and potassium increased. They concluded from their investigations that the increased potassium content of cancers is probably an expression of cellular multiplication, but that the diminution in calcium is peculiar to cancer and is partially responsible for decreased mutual adhesiveness of cancer cells, a property that is expressed in invasiveness of the surrounding tissue by cancer cells. Lansing, Rosenthal and Kamen (1948) found distinct differences in the ability of normal and carcinomatous tissues regarding the exchange of intracellular with extracellular calcium. The carcinomatous tissues were unable to exchange this metal. These authors suggest that in cancer there may be alterations in the calcium binding mechanisms, perhaps at the cell surface.

In view of my findings that " calcified " atherosclerosis is significantly less common in cancer than in non-cancer cases, it seems likely that the calcium deficiency in malignant new growth represents only a more advanced manifestation of a pre-existing systemic deviation of the tissue calcium metabolism.

Some clinical observations may be mentioned which also point to the importance of calcium in neoplasia. It is well known that in pregnancy cancer of the breast usually advances rapidly. It has been suggested that this could be due to the oestrogens and other hormones in the placenta. In view of the role of calcium deficiency in tumour growth, I would suggest that the high malignancy of breast cancer in pregnancy could be due to the drain by the foetus on the calcium reserves of the mother, this process being influenced by hormones. This explanation is in conformity with recent findings on the effect of hormones on calcium metabolism in cancer. Baker (1956) found that increases in calcium excretion, following administration of oestrogen in mammary carcinoma, are most probably associated with an increased growth rate of the tumour cells.

On the other hand a protecting influence of calcified atherosclerosis against cancer formation can be assumed in gastric ulcers of middle aged and elderly individuals. The stomach, one of the organs most commonly affected by cancer, is also frequently the site of gastric ulcer. These benign, chronic ulcers in elderly people are usually large, and even so-called giant ulcers are often non-malignant. It is, therefore, noteworthy that in the course of my investigations I found the most striking difference in the incidence and severity of calcified atheroma between gastric ulcer and carcinoma of the stomach. This is shown in Fig. 2.

In the 50–60 age group only 2 per cent of gastric carcinoma cases show calcification of the abominal aorta against 19 per cent of the controls and 61 per cent of the gastric ulcer cases. Even in the 71–80 age group this difference is still noticeable, when 23 per cent of gastric carcinoma cases against 64 per cent of the controls and 94 per cent of gastric ulcer cases show calcification of the abdominal aorta.

This striking difference in the incidence of calcification of the abdominal aorta between gastric ulcer and gastric carcinoma has been applied by me as a valuable aid for the radiological differential diagnosis of benign and malignant lesions of the stomach on many occasions.

It is surprising that these large chronic ulcers, occurring at the cancer age, frequently heal and rarely become malignant. I would suggest that it is the calcium metabolism associated with calcified atherosclerosis that protects these ulcers from malignant degeneration.

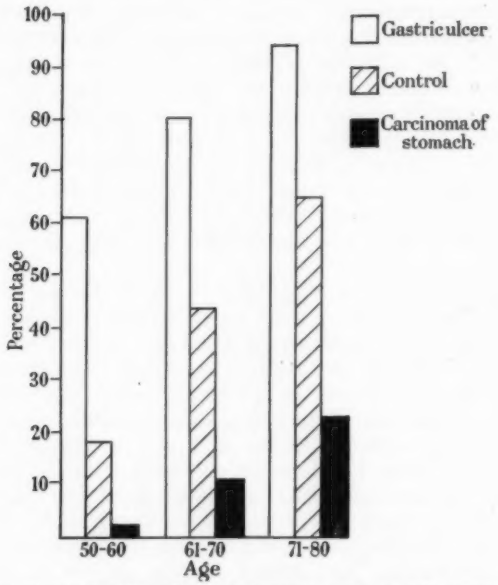

FIG. 2.—Percentage of calcification of the abdominal aorta in 133 cases of carcinoma of the stomach, 220 cases of gastric ulcer and in 1488 control cases between 50 and 80 years of age.

There is also evidence that calcified atherosclerosis may have an influence on the slow progression or latency of some tumours. This problem will be dealt with in a later paper. I would mention, however, that I observed long survival rates in a number of patients, suffering from carcinoma of the prostate, breast, kidney, colon and even of the stomach, who showed calcified atherosclerosis. In these cases calcified atherosclerosis did not prevent cancer formation, but it could be assumed that it played a part in the low malignancy of these tumours.

SUMMARY

The cancer-calcified atherosclerosis relationship has been examined in 622 cancer patients aged over 50 and in 1480 controls of the same age groups.

Radiography of the abdominal aorta was used for estimating the absence or the various degrees of calcification in the abdominal aorta.

The incidence and severity of calcified atherosclerosis is twice as high in the controls as in the carcinoma cases. These differences are even more noticeable in cancer of the sex glands, colon and stomach.

The significance of calcium salts in cancer and its possible relation to the lipoid-calcium metabolism in atherosclerosis has been discussed.

The very high incidence of calcified atherosclerosis of the abdominal aorta in gastric ulcer may explain why these large chronic lesions, occurring at the cancer age, rarely become malignant.

Carcinoma associated with calcified atherosclerosis is often of low malignancy.

The theory is advanced that a biochemical system with an increased affinity of the lipoids and proteins to calcium salts leads to calcified atherosclerosis and to a relative immunity to cancer. A biochemical system with lack of affinity to calcium salts is responsible for the absence of calcified atherosclerosis in advancing years and is a potential systemic factor in carcinogenesis.

REFERENCES

BAKER, W. H.—(1956) *Amer. J. Med.*, **21**, 5, 714.

BEEBE, S. P.—(1904–5) *Amer. J. Physiol.*, **12**, 167.

BLUMENTHAL, H. T., LANSING, A. I., WHEELER, P. A.—(1944) *Amer. J. Path.*, **20**, 665.

BRUNSCHWIG, A., DUNHAM, L. J., NICHOLS, S.—(1946) *Cancer Res.*, **6**, 230.

CLOWES, G. H. A., FRISBIE, W. S.—(1905) *Amer. J. Physiol.*, **14**, 173.

COMAN, D. R.—(1944) *Cancer Res.* **4**, 625.

DE LONG, R. P., COMAN, D. R., ZEIDMAN, I.—(1950) *Cancer*, **3**, 718.

DUNHAM, L. J., NICHOLS, S., BRUNSCHWIG, A.—(1946) *Cancer Res.*, **6**, 233.

EGGERS, H. E.—(1932) *Arch. Path.*, **13**, 296.

ELKELES, A.—(1949) *Brit. J. Radiol.*, **22**, 280.—(1950) *Int. Congr. Radiol.*, p. 78.—(1953) *Amer. J. Roentgenol*, **70**, 797.—(1956) *Brit. J. Cancer*, **10**, 247.—(1957) *Lancet*, ii, 714.

GROSSE, H.—(1958) *Z. Krebsforsch.*, **65**, 519.

LANSING, A. I., ROSENTHAL, T. B., KAMEN, M.—(1948) *Arch. Biochem.*, **19**, 177.

MC CUTCHEON, M., COMAN, D. R., MOORE, F. B.—(1948) *Cancer*, **1**, 460.

THE EFFECT OF DAILY FEEDING OF ^{90}Sr TO RABBITS

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PREVIOUS papers have discussed the amount of ^{90}Sr retained in the skeleton of rabbits following a single injection of this isotope and the pattern of its distribution (Kidman, Tutt and Vaughan, 1950, 1951; Kidman, Rayner, Tutt and Vaughan, 1952; Owen, Sissons and Vaughan, 1957). The present paper both describes the effects of continuous feeding of ^{90}Sr to rabbits and analyses the pattern and amount of isotope retention. A comparison of the damage produced by the two methods of administration and its relation to radiation dose-rate and accumulated dose will be discussed elsewhere (Owen and Vaughan, 1959).

EXPERIMENTAL METHODS

1. *Rabbits*

Dutch rabbits of the same stock and on the same diet as those in previous experiments were used for ^{90}Sr feeding (Owen, Sissons and Vaughan, 1957). This diet gave a calcium intake of 0·28 g. daily (expressed as calcium oxide) when feeding started and of 0·50 g. when the animals were 6 months old (Kidman, Tutt and Vaughan, 1950). Twenty-two of the rabbits were weanlings, i.e. 5–8 weeks old when feeding started. Two in this age group had litters during the experimental period. Four rabbits born dead from ^{90}Sr-fed mothers and two rabbits suckled for five weeks by their ^{90}Sr-fed mothers were included. Two rabbits were over 3 years old when feeding started. Some of the rabbits were killed, others died during the period of ^{90}Sr feeding. At autopsy the skeletons were dissected, weighed and in most cases radiographed. Various bones were taken for special studies described below. Knowing the weight, it was possible to allow for any bone so used in making estimations of total skeletal retention of ^{90}Sr. A variety of soft tissues including representative samples of the various parts of the intestinal tract, as well as liver, spleen, kidneys and gonads, were examined histologically.

2. *Method of feeding ^{90}Sr*

^{90}Sr was fed incorporated in an M.R.C. pellet. The animals were starved overnight and fed a ^{90}Sr pellet each morning to ensure ingestion. In addition to the ^{90}Sr fed in the pellet, there was what might be termed secondary ingestion, due to the rabbit's habit of coprophagy. Intake from this is not included in the ^{90}Sr intake figures.

It would theoretically have been more satisfactory to have given the rabbits all their food in the form of pellets containing ^{90}Sr so that the $^{90}Sr/Ca$ ratio in the diet could have been kept constant throughout the experiment. The pellet diet,

however, has a higher calcium content than the oats, hay and greenstuff diet which had been given in other experiments (Kidman *et al.*, 1950; Owen, Jowsey and Vaughan, 1955; Owen, Sissons and Vaughan, 1957), and the experimental results obtained here would not therefore have been comparable.

3. *Preparation of ^{90}Sr pellets*

A known weight and number of M.R.C. pellets was moistened with distilled water. To this was added a solution of ^{90}Sr in 0·1 N HCl which was calculated to give 10 μc ^{90}Sr per pellet. The paste was then made into pellets. Although every effort was made to mix the paste thoroughly and to make the pellets to constant size there was inevitably some slight variation. For instance a sample of 15 pellets from one batch had a mean value of 8·5 μc per pellet, while a sample of 20 pellets from another batch had a mean value of 11·8 μc per pellet. The other batches fell between 8·0 and 11·8 μc per pellet. A further batch of pellets was made up using a solution of higher activity which gave a mean content of 25 μc. It was known which batch was fed to each rabbit so that the mean value of the batch was taken into account in calculating total intake.

4. *Estimation of ^{90}Sr and calcium*

The bones were dissolved in nitric acid, and ^{90}Sr retention measured by methods described elsewhere (Kidman, Tutt and Vaughan, 1950). Aliquots equivalent to about 0·03 g. bone were evaporated to dryness and ashed, and their calcium content estimated by the method already described (Sheldon-Peters and Vaughan, 1956).

The figure for ^{90}Sr fed was calculated from the ^{90}Sr content of the pellets and the number of pellets eaten from each batch. The ^{90}Sr content of the pellets was known to within $\pm$ 3 per cent, except when the number of pellets fed was less than 5, when the error became $\pm$ 10 per cent. Since the whole skeleton could not be used for ^{90}Sr estimation, the figures for its total retention was calculated from the measured amount in those parts available. The percentage analysed is stated.

The figures at the top of each column in the tables are standard deviations on the analytical methods with the exceptions of those involving the weights of tibiae or skeletons. The difficulty of weighing wet bones reproducibly makes the results in these columns reliable within an estimated range of $\pm$ 7 per cent in the case of tibiae and $\pm$ 20 per cent in the case of skeletons.

5. *Haematological and histological techniques*

Haematological and histological techniques were those used in previous work (Owen, Sissons and Vaughan, 1957). Platelets were estimated from examination of stained films. The standard deviation on the haemoglobin value of normal rabbits in the younger age group in this laboratory is 0·88 and on the total white cell count 1560. In the older age group the haemoglobin standard deviation is 0·65 and for the white count 957.

6. *The use of the tibia*

Since the pattern of growth has been studied in more detail in the tibia than in any other bone (Owen, Jowsey and Vaughan, 1955), the two tibiae of each rabbit were used for several different purposes. The right tibia was first used for

radiation dosimetry measurements which are reported in detail elsewhere (Owen and Vaughan, 1959). For this purpose it was necessary to cut the proximal half of the bone into two longitudinal halves for the preparation of contact autoradiographs needed for dosimetry measurements (Lamerton and Harriss, 1954; Sinclair, Abbatt, Farran, Harriss and Lamerton, 1956). The two halves were then stuck together and thin cross-sections cut for the preparation of autoradiographs and microradiographs. The left tibia was used either for chemical analysis of ^{90}Sr and Ca content or for histological examination.

7. *Microradiographs and autoradiographs*

Microradiographs and autoradiographs were prepared from thin cross-sections of the tibia and from the mandible, as previously described (Jowsey, 1955; Owen, Jowsey and Vaughan, 1955). Kodak Scientific Plate autoradiographic stripping film (AR10) was used except when the low ^{90}Sr content of bone made the use of Kodak Scientific Plate fast autoradiographic stripping film (AR50) necessary.

RESULTS

I. *General Effects of ^{90}Sr*

The effect of feeding 8–11·8 μc ^{90}Sr daily on the weight and the blood picture of 8 rabbits that were 5–8 weeks old when feeding started, and of two much older rabbits, are shown in Table I, together with the amount of ^{90}Sr fed and the amount retained in the skeleton in 14 rabbits and in six progeny born during the exerimental period. Figures for the amount of ^{90}Sr fed and retained in 9 rabbits fed 25 μc daily are shown in Table II.

Soft Tissues

Sections of lungs, liver, spleen, kidneys, adrenals and gonads, and of the walls of various parts of the alimentary canal, failed to show histological evidence of any important radiation damage. In some instances, post-mortem changes were present and may have obscured any minor abnormalities of the alimentary mucous membranes, but neither frank ulceration nor scarring of the intestinal wall was encountered. In rabbits dying in the first few weeks of ^{90}Sr feeding, histological evidence of damage to bone marrow was slight, being restricted to some loss of haemopoietic cells in the metaphyseal regions of the long bones. In rabbits dying later in the experiment, i.e. more than 200 days after feeding started, bone marrow was fatty and atrophic although areas of active haemopoiesis could always be found. Mucoid degeneration of bone marrow was found in a small area in only one animal (925).

II. *Effects on the Skeleton*

1. *General*

Weanling rabbits fed 8–11·8 μc daily.—No gross skeletal abnormalities were noted in the skeletons of weanling rabbits fed for 3 to 48 days. Gross skeletal abnormalities were observed in 4 (922, 925, 930, 932) of the 5 rabbits which survived more than 200 days from the time feeding started at 5–8 weeks.

Two of these rabbits (922 and 932) developed multiple bone tumours which were evident by general inspection during life, and by radiographic examination

TABLE I.—*The Effect on the Skeleton, Haemoglobin, White Blood Count, Platelets and Weight of Daily Feeding of 8–11·8 μc ^{90}Sr*

Rabbit No.	Age ^{90}Sr feeding began (days)	Initial weight (g.)	^{90}Sr feeding days	^{90}Sr fed μc. ±3%*	Skeleton ^{90}Sr: Total μc.* ±20%	Skeleton ^{90}Sr: μc./g. Ca* ±3%	Skeleton ^{90}Sr: μc./g. bone ±20%*	Tibia ^{90}Sr: Total μc. ±3%*	Tibia ^{90}Sr: μc./g. Ca ±3%*	Tibia ^{90}Sr: μc./g. bone ±7%*	Tibia ^{90}Sr: g. Ca/g. bone* ±7%	% skeleton analysed	W.B.C. (thousands c.mm.): Before ^{90}Sr feeding	W.B.C. (thousands c.mm.): Terminal	Hbg. %: Before ^{90}Sr feeding	Hbg. %: Terminal	Platelets	Wt. loss (g.)	Remarks
1108	45	620	3	35	19	2·9	0·17	0·71	2·9	0·29	0·103	54	—	—	—	—	..	—	Killed
1110	46	690	5	59	17	3·0	0·14	1·08	3·1	0·26	0·084	75	—	—	—	—	..	—	,,
1112	46	620	5	59	13	2·4	0·12	1·08	3·8	0·38	0·102	71	—	—	—	—	..	—	,,
1109	45	740	9	106	25	3·3	0·18	1·57	7·5	0·46	0·061	75	—	—	—	—	..	—	,,
1111	45	620	9	106	24	5·1	0·19	1·46	7·7	0·52	0·67	87	—	—	—	—	..	—	,,
973	44	650	31	248	130	12·9	1·1	4·5	10·8	1·6	0·15	45	4600	3700	12·6	11·1	..	0	Died (accident)
974	44	550	31	248	100	9·3	0·8	3·0	6·9	1·0	0·15	55	5100	6400	13·3	13·3	..	0	Killed
924	52	810	48	440	—	—	—	—	—	—	—	—	5000	4600	12·0	11·8	R	0	,,
930	52	730	224	1870	220	14·0	1·1	12·3	15·6	2·7	0·18	95	6600	6800	13·0	5·8	,,	50	Died
931†	54	780	240	2020	150	8·8	0·9	9·6	11·7	2·0	0·17	75	5500	2600	13·8	11·2	,,	50	Killed
925‡	54	890	252	2060	190	10·6	1·3	12·4	13·5	2·7	0·20	70	4200	1700	12·4	4·6	,,	730	Died
932	52	730	263	2060	220	13·2	1·1	9·2F	6·9	1·5	0·21	60	4000	1700	13·3	13·0	S	10	,,
922	54	890	280	2070	250	13·0	1·5	13·0F	13·5	2·2	0·16	50	3100	2000	11·8	8·8	R	620	,,
925*a*	F foetus	..	..	..	0·23	0·81	0·029	0·0066	0·95	0·077	..	..	..	..	..	..	..	..	Born dead
925*b*	,,	..	..	..	0·18	0·90	0·019	0·0074	0·96	0·074	..	..	..	..	..	..	..	..	,, ,,
931*a*	,,	..	..	..	0·20	0·85	0·028	0·0047	0·52	0·060	..	..	..	..	..	..	..	..	,, ,,
931*b*	,,	..	..	..	0·19	0·91	0·025	0·0044	0·61	0·051	..	..	..	..	..	..	..	..	,, ,,
931*c*	0	..	35	..	8·9	1·75	0·10	0·49	1·95	0·23	..	..	..	..	..	..	..	..	Killed
931*d*	0	..	35	..	10·5	1·96	0·10	0·61	2·13	0·25	..	..	..	..	..	..	..	..	,,
921	4 years	..	162	1300	8·1	0·35	0·031	0·126	0·126	0·022	0·17	95	4400	8600§	10·9	10·5	S	12	Died
918	3¼ years	..	431	6750°	130	..	..	2·00	1·99	0·33	0·17	2	5700	2400	11·8	11·0	,,	6	Killed

F = femur.
† = had a litter, 2 dead, 2 suckled 5 weeks : *a*, *b*, *c*, *d*
‡ = had a litter, 2 dead : *a*, *b*.
R = reduced.
S = steady level.

° This rabbit received some pellets containing 25 μc. ^{90}Sr.
§ rose as high as 26,000.
* see page 3.

EXPLANATION OF PLATES

FIG. 1.—Rabbit 932, fed ^{90}Sr for 263 days from the age of six weeks—radiograph. Note: (i) tumour of upper end of left tibia associated with increased density; (ii) tumour of mandible.

FIG. 2.—Rabbit 922, a longitudinal section from the upper part of the humerus. Tumour tissue is present in both the epiphysis and metaphysis, and projects as a large subperiosteal mass. The tumour is an osteosarcoma, with relatively little bone formation. × 3.

FIG. 3.—Rabbit 922, transverse section of skull, showing tumour tissue surrounding the roots of the maxillary molar teeth on each side. The lesions are osteosarcomas, and appear to have had origin in the bone of the tooth sockets. × 1¼.

FIG. 4.—Rabbit 922, a longitudinal section of the upper part of the tibia, showing complete replacement of marrow by bony tumour tissue. The posterior cortex is invaded in several places, and a subperiosteal extension is present. × 3.

FIG. 5.—Rabbit 932, a longitudinal section of the lower part of the femur. Tumour tissue occupies the lower end of the bone and appears to be extending upwards in the marrow cavity. Note the contrasting appearance of the tumour tissue in different parts of the lesion. × 1¾.

FIG. 6.—Rabbit 932, a longitudinal section from the upper part of the humerus. The marrow cavity is occupied by a mass of cellular tumour tissue containing some vascular cysts. The lesion is an osteosarcoma with inconspicuous bone formation. × 1¾.

FIG. 7.—Rabbit 922, the highly differentiated osteoblastic tumour tissue comprising the subperiosteal mass seen in Fig. 4. × 43.

FIG. 8.—Rabbit 922, ossifying tumour tissue occupying marrow spaces from the upper part of the lesion seen in Fig. 4. × 43.

FIG. 9.—Rabbit 922, pleomorphic tissue from the advancing margin of the tumour seen in Fig. 4. × 43.

FIG. 10.—Rabbit 932, cellular tissue with inconspicuous strands of tumour bone from the lower part of the lesion shown in Fig. 5. × 63.

FIG. 11.—Rabbit 932, highly differentiated tumour bone forming a network between fat cells of the marrow, from the upper part of the lesion shown in Fig. 5. × 33.

FIG. 12.—Rabbit 932, the upper margin of the lesion shown in Fig. 5, tumour cells are extending between the fat cells of the marrow and are forming a network of tumour bone which appears as darkly stained material in the lower part of the field. × 30.

FIG. 13.—Rabbit 932, one of the nodular masses of cellular tumour tissue from the central part of the lesion in Fig. 5. × 30.

FIG. 14.—Rabbit 932, unusual new bone formation (? highly differentiated tumour tissue) continuous with the lower part of the lesion shown in Fig. 6. × 30.

FIG. 15.—Rabbit 932, another field showing the more detailed structure of tissue similar to that shown in Fig. 13. × 56.

FIG. 16.—Rabbit 932, part of an area of active bone formation (? early tumour) which is evident in the metaphysis in Fig. 5 above and separate from the main lesion. × 43.

FIG. 17.—Rabbit 925, longitudinal section of mid-shaft of tibia, showing a mass of osteoblastic tumour tissue which appears to be continuous with a layer of newly formed bone on the endosteal surface of the shaft. × 9.

FIG. 18.—Rabbit 922, section through the base of a mandibular molar tooth, showing erosion of dentine by a localised area of proliferating osteoblastic tissue (? early tumour). × 45.

FIG. 19.—Rabbit 932, the lower margin of the unusual bone tissues shown in Fig. 14 and 15. Note the resemblance to Fig. 12. × 57.

FIG. 21.—Rabbit 932, fed ^{90}Sr for 263 days from the age of six weeks. Section 17. Autoradiograph. Note: (i) uneven uptake of ^{90}Sr in bone formed after feeding started, the more recently formed bone on the endosteal and periosteal surfaces containing less ^{90}Sr. (ii) High ^{90}Sr content of certain Haversian systems. (iii) Abnormal endosteal bone in lower right-hand corner. × 5.

FIG. 22.—Rabbit 921, fed ^{90}Sr for 162 days from the age of 4 years. Section 47—upper end of tibia. Autoradiograph. Note heavy uptake of ^{90}Sr on edges of trabeculae, patchy in distribution. × 40.

FIG. 23.—Rabbit 921, fed ^{90}Sr for 162 days from the age of 4 years. Section 19—upper half of tibia. Autoradiograph. Note: (i) heavy, though patchy, uptake of ^{90}Sr on endosteal surfaces. (ii) Occasional heavy periosteal uptake of ^{90}Sr.

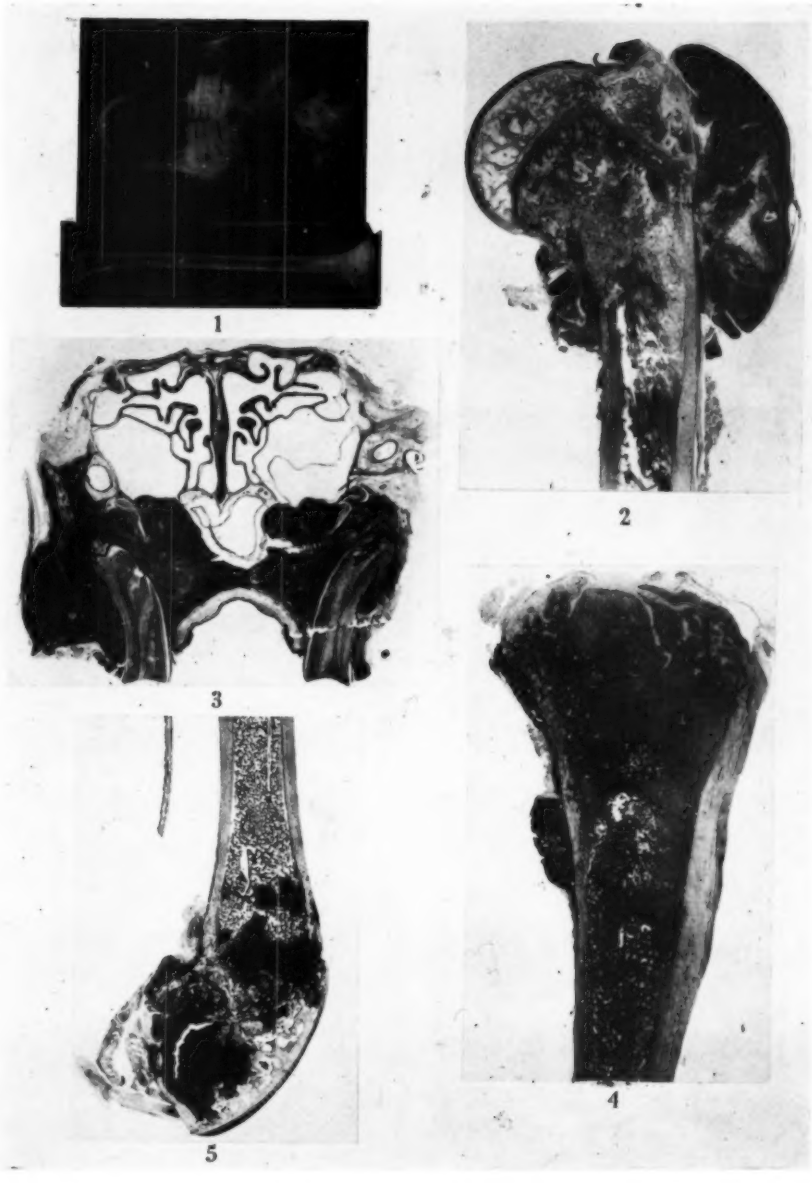
FIG. 24.—Rabbit 932, fed ^{90}Sr for 263 days from the age of six weeks. Section 42 upper half of tibia; microradiograph of posterior-medial corner. Note: abnormal bone on endosteal surface, possible osteosarcoma. × 21.

FIG. 25.—Rabbit 932, fed ^{90} Sr for 263 days from the age of six weeks. Section 28—upper half of tibia; microradiograph. Note: (i) osteosarcoma extending along part of posterior, medial and lateral endosteal surface. (ii) The bands of white are the sites where the bone was cut to prepare gross autoradiographs. Compare with Fig. 11. × 6.

FIG. 26.—Rabbit 932, fed ^{90}Sr for 263 days from the age of six weeks. Section 15—upper half of tibia, microradiograph of lateral-medial corner. Note: abnormal endosteal bone, large lacunae, irregular lamellae, irregular surface. × 24.

FIG. 27.—Rabbit 932, fed ^{90}Sr for 263 days from the age of six weeks. Section 25—upper half of tibia; microradiograph. Note: abnormal buried periosteal bone showing large lacunae and irregular arrangements of the lamellae. × 22.

FIG. 28.—Rabbit 918, fed ^{90}Sr for 431 days from the age of 3·5 years. Autoradiograph of molar teeth and adjacent mandible. Note relatively low ^{90}Sr uptake in bone and high uptake throughout teeth. × 4.



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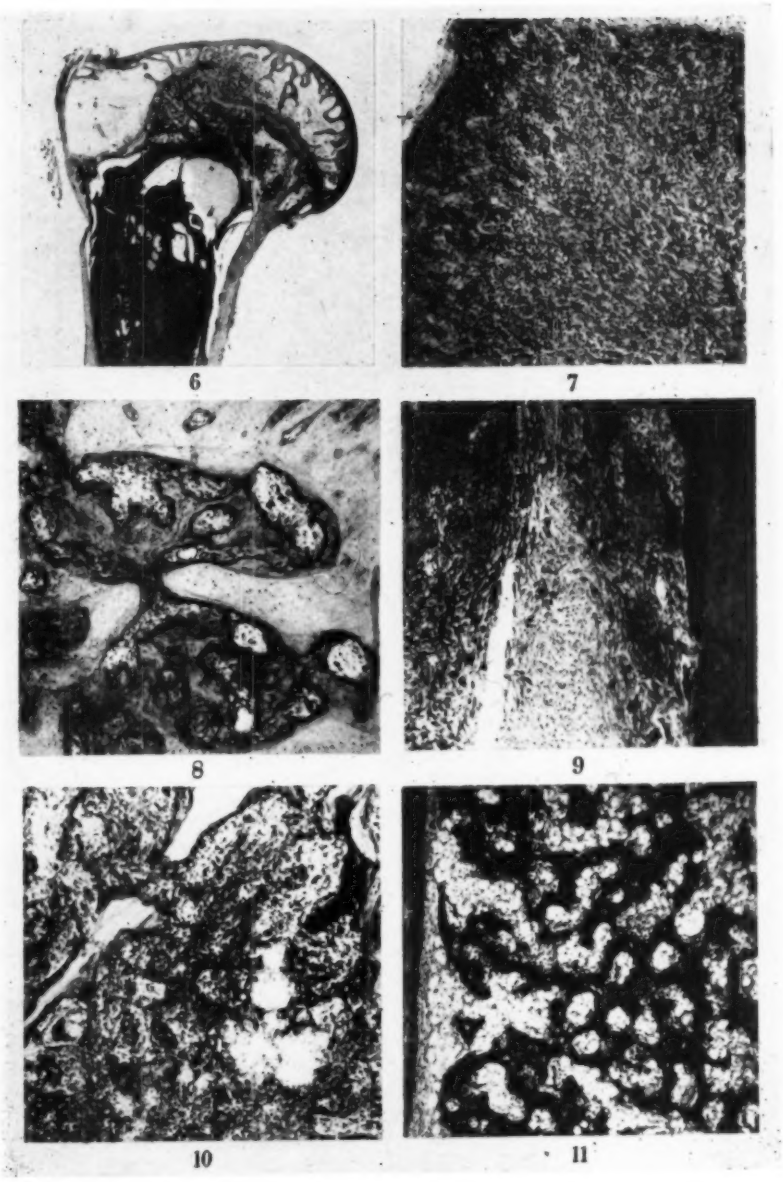

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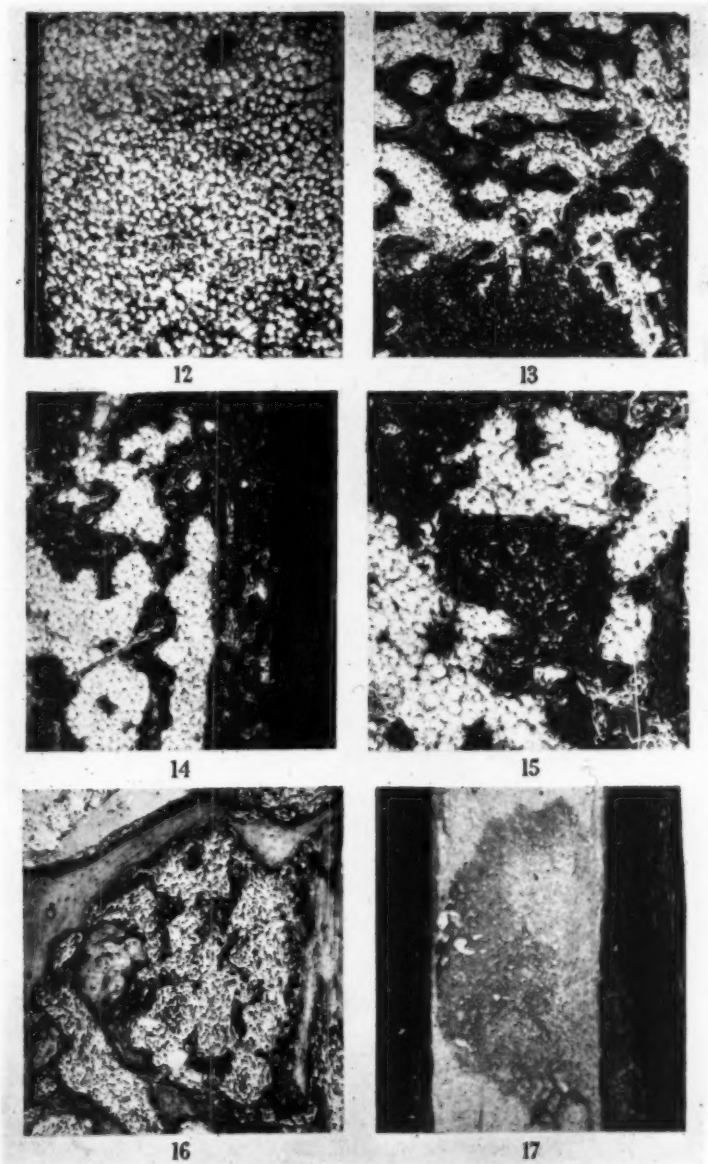

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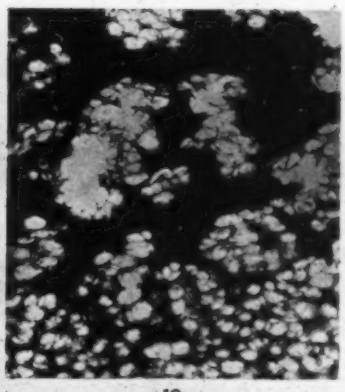

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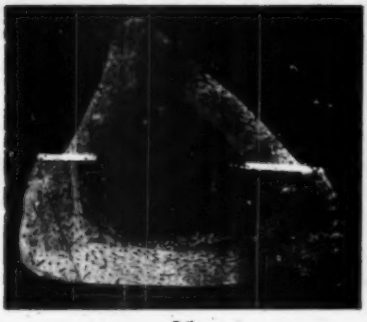

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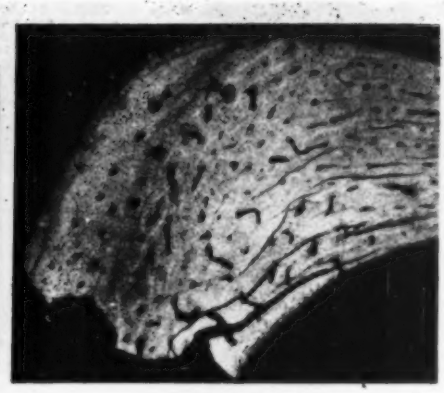

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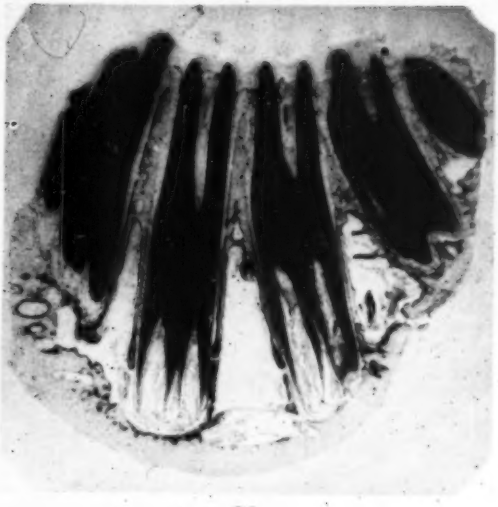

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TABLE II.—*The Effect on the Skeleton of Daily Feeding of 25 μc of ^{90}Sr*

Rabbit No.	Age ^{90}Sr feeding began (days)	Initial weight (g.)	^{90}Sr feeding days	^{90}Sr fed μc. ±3%	Skeleton ^{90}Sr Total μc. ±20%	Skeleton ^{90}Sr μc./g. Ca +3%	Skeleton ^{90}Sr μc./g. bone ±20%	Tibia ^{90}Sr Total μc. ±3%	Tibia ^{90}Sr μc./g. Ca ±3%	Tibia ^{90}Sr μc./g. bone ±7%	Tibia ^{90}Sr g. Ca/g. bone ±7%	% skeleton analysed	Remarks
1076	42	620	3	74	25	4·6	0·19	1·35	5·7	0·52	0·090	58	Killed
1081	48	470	3	74	20	6·5	0·20	1·15	5·9	0·60	0·103	46	,,
1072	43	600	5	123	45	7·9	0·32	3·4	15·3	1·26	0·084	53	,,
1074	43	610	5	123	64	12·1	0·5	4·1	17·0	1·5	0·099	52	,,
1075	43	640	9	221	89	15·8	0·6	6·0	22·1	1·9	0·085	56	,,
1085	48	680	9	221	100	14·8	0·8	3·9	14·0	1·4	0·099	50	,,
1044	49	620	9	221	61	6·6	0·33	3·7	11·9	1·15	0·099	75	,,
994	42	450	40	985	215	36	1·2	11·1	31	3·1	0·098	70	Died
1102	45	460	40	985	230	40	2·1	10·6	44	4·6	0·104	57	Killed

of the skeleton. These tumours were seen at the rapidly growing ends of the long bones and in the skull (Fig. 1, 2).

Two other rabbits (930 and 925) had gross tumours of the mandible which were evident at post-mortem. One rabbit (931) had no gross tumours, either at post-mortem or on radiographic examination, but osteosarcoma of the mandible was found on histological examination. Apart from obvious tumours, radiographic examination showed generally increased density of the epiphysis, metaphysis and adjacent diaphysis at the rapidly growing ends of the long bones in 3 rabbits. Histological examination showed that lesions of this type were osteosarcomas, as was the case in the " bar " region in the injection experiment (Owen, Sissons and Vaughan, 1957).

There was no difference between the pellet-fed animals and their controls with regard to the size and weight of bones. It was noticeable that in none of the bones studied was there significant interference with bone resorption and remodelling.

No appreciable amounts of endosteal or peritrabecular fibrosis, of the sort found in rats receiving plutonium (Lisco, 1956) or in dogs receiving other alpha-emitting bone-seeking isotopes (Jee, Arnold and Cochran, 1957) were encountered in the present material.

Old rabbits

No gross skeletal abnormalities were found in the skeleton of the two old rabbits (921, 918) fed for 162 and 431 days respectively. The only histological change noted was the presence of scattered dead osteocytes, particularly in cortical bone, in rabbit 918.

2. *Tumours*

As already noted, each of the 5 weanling animals fed for a long period developed bone tumours, which, whenever the skeleton was studied in detail, were found to be multiple. The usual sites were the bone surrounding both the maxillary and mandibular molar teeth (Fig. 3), the upper end of the tibia (Fig. 4) and the lower end of the femur (Fig. 5), and to a lesser extent the upper end of the humerus (Fig. 2) and the upper end of the femur. Tumour tissue either extended diffusely throughout the marrow cavity of the involved bone (Fig. 4), or invaded the cortex and produced an external mass (Fig. 2). The tumours of the long bones appeared always to arise from the endosteal surface but it was usually impossible to be more precise with regard to their exact sites of origin (see section on the tibia.) As in the injection experiments, the tumours could all be described as osteosarcomas (Fig. 2, 3, 7, 8, 9, 10, 11, 12, 13). Some contained very little tumour bone : in others this was a very conspicuous feature. None of the tumours so far studied presented any tumour cartilage.

Many of the tumours showed the same range of histological structure encountered in human osteosarcomas : some, however, (and this was also a feature of the tumours encountered in the injection experiment) showed an unusually high degree of structural differentiation, conspicuous tumour bone with a remarkably mature histological appearance being present. This feature is emphasised in the lesions chosen for illustration in the present paper, and is shown in Fig. 7, 8, 11, 14, 15.

In addition to established tumours, which would be identified as osteosarcomas

by all experienced pathologists, we encountered a few lesions whose nature was not so clear. These took the form of localised foci of proliferating osteoblastic tissue, and were similar to those encountered in the injection experiment (Owen, Sissons and Vaughan, 1957) and then described as early sarcomas or " presarcomatous " lesions. In the present material these were found in the long bones (Fig. 16, 17) but were also encountered in relation to the bone surrounding mandibular and maxillary molar teeth (Fig. 18). It is necessary to be sure that some or all of these small lesions are not merely extensions from larger established tumours : while absolutely rigorous proof of this would demand complete serial sectioning of the involved bones, it can be stated that foci of this sort were encountered in sections in which there was no evidence of gross tumours. One interesting and apparently early lesion is the nodule of tumour occupying the marrow cavity of the mid-shaft of the tibia in rabbit 925 (Fig. 17). This consists of proliferating osteoblastic tissue, and appears to be continuous with a layer of newly formed bone that is present on the endosteal surface of the shaft in this situation.

An interesting histological feature of some of the established tumours was the type of regional variation in histological structure that is illustrated by the lesions shown in Fig. 5, 10, 11, 12, 13 and Fig. 6, 14, 15, 19. In Fig. 5 the lower end of the femur of rabbit 932 is occupied by a mass of cellular, invasive and obviously malignant tumour tissue : the shaft, in contrast, is occupied by a network of highly differentiated bone tissue (Fig. 11) which can be recognised as invasive only at its upper margin where tumour cells are infiltrating the spaces between fat cells of the marrow (Fig. 12). Do these contrasting areas represent independent tumours, or parts of the same lesions? If the latter, what is the relationship between them? A similar problem is posed by the existence, within the humerus of the same rabbit (932), of a number of different types of apparently neoplastic tissue (Fig. 6, 14, 15, 16, 19). The largest of these (Fig. 6) consists of a central mass of frankly malignant tumour tissue showing little new bone formation. Above this (Fig. 16), there is a patch of apparently separate osteoblastic tissue : this is interpreted as representing an independent neoplastic focus. But below the main lesion, and continuous with it, is a most unusual type of osteoblastic tissue (Fig. 14, 15), which, though cellular, can only be recognised as invasive at its lower margin (Fig. 19). Here, as with the tumour of the femur, the relationship between the two contrasting areas of tumour tissue is of interest.

3. *A study of the upper half of the tibia*

As in previous work on normal rabbits and rabbits given a single injection of ^{90}Sr the upper half of the tibia has been studied in much greater detail than any other bone. The distribution of the retained ^{90}Sr as seen on autoradiographs of thin sections and the radiation damage as seen on microradiographs and some histological sections is discussed here. The results of the measurement of radiation dose-rate and its relation to damage in different parts of the bone are described in detail elsewhere (Owen and Vaughan, 1959).

(i) *Distribution of ^{90}Sr. Weanling rabbits.*—The results are best described with reference to Fig. 20 which shows 2 diagrams of the upper half of the tibia of an adult rabbit (i.e. aged 6 months plus). In Fig. 20*a* are shown in black the main regions of ^{90}Sr retention in a rabbit fed ^{90}Sr from the age of 5–8 weeks. In Fig. 20*b* is shown the pattern of bone growth after the age of 5–8 weeks. It will be seen that ^{90}Sr is taken up throughout the bone formed after ^{90}Sr feeding started.

There is a slight simplification here since some ^{90}Sr is also always taken up in remodelling Haversian systems in the bone which existed before feeding started For a detailed description of the pattern of growth of the upper half of the normal

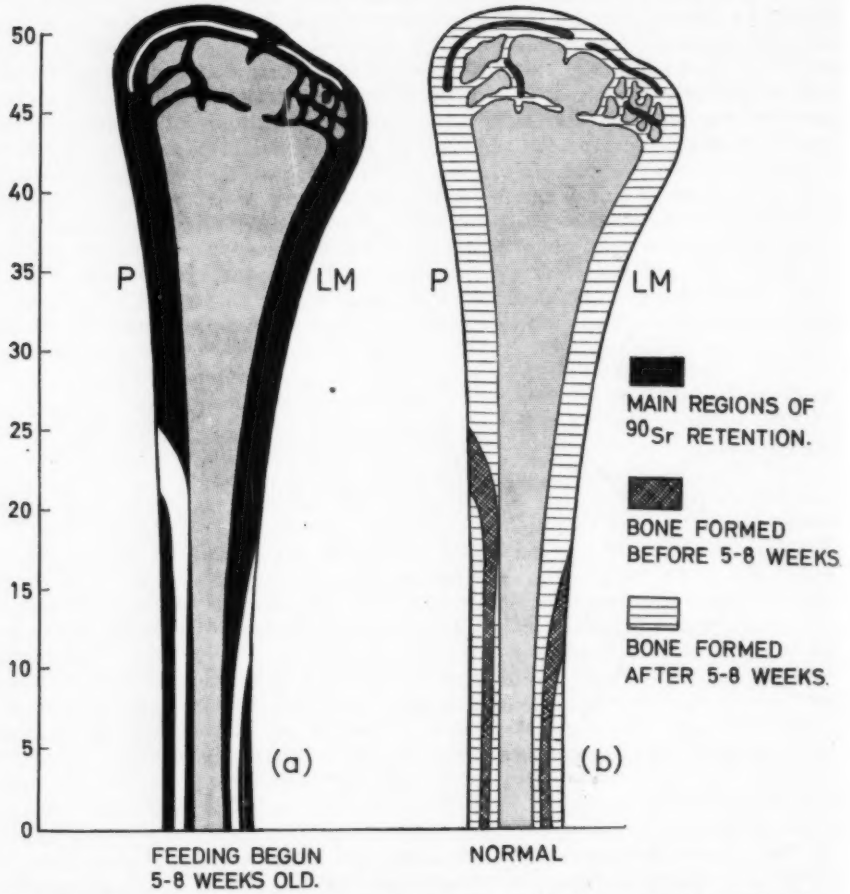

FIG. 20.—Diagrams of longitudinal section through the middle of the posterior wall P and the lateral medial corner LM of the upper half of the tibia of a fully-grown rabbit, showing main regions of ^{90}Sr retention in relation to bone growth.

rabbit tibia, reference should be made to earlier work (Owen, Jowsey and Vaughan, 1955). The numbers at the side of the diagram refer to the approximate levels of sections, mentioned in the text, from which microradiographs and autoradiographs were made.

It was found, however, that in the animals which survived for 200 days or more, the most recently-formed bone contained less ^{90}Sr than that which was

formed soon after ^{90}Sr feeding started. This point is illustrated in Fig. 21, where the most recently-formed bone (an endosteal band, lower right, and a periosteal band, upper left) shows a lower content of ^{90}Sr than the greater part of the bone formed during the course of the experiment. There was also some variation in ^{90}Sr content in different Haversian systems associated with variation in their calcium content as seen in microradiographs. Tumours containing calcified tissue held some ^{90}Sr but much less than adjacent bone.

Old rabbits.—In these two rabbits, which died 162 and 431 days after starting ^{90}Sr feeding, there was a diffuse distribution of ^{90}Sr throughout the bone but in extremely low concentration. Heavy patchy uptake was seen on the edges of bone trabeculae and on the endosteal surfaces in the epiphysis (Fig. 22). Throughout the shaft there were local concentrations on the endosteal surface (Fig. 23) and occasionally on the periosteal surface and in Haversian systems.

Offspring of rabbits fed ^{90}Sr.—Autoradiographs were not made on bone sections from the rabbits suckled for five weeks. In the rabbits born dead the whole tibia contained ^{90}Sr but this was in higher concentration in the bone in the middle third than it was in the cartilage at either end.

(ii) *Radiation damage. Weanling rabbits.*—Microradiographs of cross-sections of the upper half of the tibia of the rabbits killed or dying less than 31 days after feeding started showed in general no abnormality. The tibia of rabbits fed for over 200 days showed considerable evidence of radiation damage. In some, extensive and obvious tumour extended from the epiphysis into the mid-diaphysis ; in others tumour was present but not so extensive. There was abnormal bone, particularly on the endosteal surface of the medial and posterior walls. This abnormal bone appeared in the microradiographs of cross-sections to have large lacunae and an extremely irregular appearance of the bone lamellae, while the endosteal surface showed varying degrees of irregularity. It extended along the length of the bone studied and it was impossible to say at which level it had originated. Such bone can be seen in Fig. 24 in the posterior-medial corner at level 42. This figure also illustrates the extreme irregularity of the surface suggesting that there is possibly early osteosarcoma. Fig. 25 shows a cross-section at level 28 from the same bone, at which level there was great proliferation of abnormal tissue, which could be seen to fill the whole cavity when the section was cut. It was not all calcified and therefore does not show in the microradiograph. When stained, this section had an appearance like that seen in Fig. 12. There was highly differentiated tumour bone between the fat cells of the marrow. Fig. 26 shows a high power view of the endosteal surface in the medial-lateral corner at level 15, again from the same bone. Here are seen again the abnormal enlarged lacunae and an irregular surface. Histological preparations from such a site showed the lacunae to be empty and the bone to stain poorly, while the irregular surface was often associated with osteosarcomatous tissue showing varying degrees of bony differentiation.

Other changes took the form of scattered but patchy loss of osteocytes, which was sometimes, but not always, associated with damage to small blood vessels. The cell loss was often evident in the compact bone of the shafts of the long bones. In different animals and in different bones, the intensity of this change varied from minimal to obvious. The vascular changes took the form of occlusion of small blood vessels in Haversian spaces by fibrin clot, or were evident from an absence of any stainable vascular structures in the areas of bone damage.

The changes seen in microradiographs in bone formed on the periosteal surface were always less dramatic than those seen in microradiographs of endosteal bone. In no instance was tumour seen associated with the periosteal surface only, and in no instance was this surface irregular. The type of change in periosteal bone is shown in Fig. 27, a microradiograph from a section at level 25 (rabbit 932). It can be seen that buried some way below the present periosteal surface, there is a band with irregular arrangement of bone lamellae associated with enlarged lacunae. In the corresponding autoradiograph this area of bone is associated with the heaviest ^{90}Sr concentration. In histological preparations at the same level the large lacunae contain no osteocytes, there may be some vascular damage and the bone stains poorly.

In the animal fed 25 μc daily which died 40 days later microradiographs showed areas of extremely low calcification particularly in lamellar bone and some abnormal periosteal bone. The metaphyseal trabeculae were devoid of active osteoblasts and there appeared to be an excessive amount of osteoclastic activity.

Old rabbits.—No radiation damage was noted in the microradiographs of the tibiae of these animals.

III. *Teeth*

Observations on the uptake of ^{90}Sr in the teeth, as seen in autoradiographs have been made on only a few animals. In a weanling rabbit killed 9 days after feeding started there was an even uptake of ^{90}Sr in the roots of the teeth and in bone that had probably been laid down in that period. In rabbit 918, aged $3\frac{1}{2}$ years when feeding started and fed for 431 days, the mandible showed only slight patchy uptake characteristic of old bone but the teeth showed an even heavy uptake of ^{90}Sr throughout their length (Fig. 28). This is in agreement with the fact that there is very little growth or remodelling in the bone of an animal of this age, whereas the teeth of the rabbit continue to grow throughout life. There was no histological damage in this case in either jaw bone or teeth, but the development of tumour in relation to the base of the molar teeth in other animals which were younger when feeding started has already been illustrated in Fig. 4 and the appearance of abnormal proliferating osteoblastic tissue eroding the dentine in Fig. 22.

DISCUSSION

The present results indicate that, in weanling rabbits fed 8–11·8 μc ^{90}Sr daily, multiple osteogenic sarcomas develop within 6–8 months in the rapidly growing ends of the long bones and the skull. Such tumours in the long bones are associated with abnormal endosteal bone surfaces extending from the epiphysis into the upper part of the diaphysis and with varying degrees of marrow aplasia. The widespread distribution of radiation dysplasia is associated with a widespread distribution of ^{90}Sr in all bone formed after feeding started. This picture differs from that seen when ^{90}Sr is given in a single injection, when there is only a localised region of intense radiation dysplasia in the same rapidly growing ends of the long bones which often become malignant, and show a high local concentration of ^{90}Sr.

The relationship of radiation dose-rate and radiation damage in the two groups

of animals is discussed in detail elsewhere (Owen and Vaughan, 1959) and has already been briefly summarised (Vaughan and Owen, 1959). Certain other points of interest are discussed below.

1. *The significance of the changes noted in the blood and in the haemopoietic organs*

The peripheral blood picture in rabbits fed for longer than 200 days was that associated with varying degrees of marrow aplasia ; in some cases the haemoglobin level was more affected, in others the white cells. In no instance was a true leukaemic blood picture seen, though a white cell count of 27,000 per c.mm. without any increase of abnormal white cells was once found in one rabbit ; in no instance was any blood loss noted. Though in some cases the terminal anaemia and leucopaenia were severe, a general gelatinous degeneration of the marrow was not found. In some cases patches of such gelatinous degeneration were present but the usual picture was of aplastic fatty marrow containing considerable areas of active haemopoiesis. The direct cause of death in all the younger rabbits at least was probably a haemopoietic one due to irradiation of the marrow by the ^{90}Sr contained in the skeleton.

2. *The histogenesis of the bone tumours*

General factors concerning the production of bone tumours by radioactive materials and their histogenesis were considered in our previous account of the effects of injected ^{90}Sr (Owen, Sissons and Vaughan, 1957). The tumours developing in the present experiment are very similar to those encountered previously. They are all osteosarcomas, and contain a variable amount of tumour bone. Their histological structure is generally comparable with that of human osteosarcomas, although some show an unusually high degree of structural differentiation.

Despite the clearly malignant local characteristics of the tumours, no metastases were observed in the present animals. In this connection it is of interest that only one of eight tumour-bearing animals in the injection experiment (Owen, Sissons and Vaughan, 1957) showed metastases, which were present in the liver. At first sight, this is in contrast with general experience with bone tumours produced with radioactive materials, whether in man (Martland, 1931) monkeys (Petrov *et al.*, 1952), rabbits (Schürch and Uehlinger, 1935 ; Ross, 1936 ; Hellner, 1937) or rats (Kuzma and Zander, 1957*a*) where metastases have frequently been observed. We believe, however, that in common with other radiation-induced bone tumours, those produced by ^{90}Sr in rabbits are highly malignant, and that our animals, which were killed when bone tumours were recognised, or which died from other causes, would have developed metastases if they had survived longer.

With regard to the histogenesis of the tumours, the early lesions observed in our present material are in keeping with our suggestion (Owen, Sissons and Vaughan, 1957) that the tumours develop from the osteogenic connective tissue covering the surfaces of areas of abnormal bone. Proliferative " presarcomatous lesions " or " early sarcomas " of the type we reported in rabbits injected with ^{90}Sr have been observed in the present material : they have also been reported by Litvinov (1957) in rats injected with ^{90}Sr, and by Kuzma and Zander (1957*b*)

in rats injected with ^{45}Ca or ^{90}Sr. While their frequent occurrence in animals receiving high dosage of radioactive isotopes, and their histological similarity to more established tumours suggests very strongly that they represent a stage in the development of the later lesions, final decision regarding their exact nature and significance must still be deferred. Information on the dose-dependence of these proliferative lesions, and of other changes such as the peritrabecular fibrosis observed with alpha-emitting bone-seeking isotopes (Lisco, 1956; Jee, Arnold and Cochran, 1957) may clarify their relationship to tumour formation. If, for example, it is found that tumours develop at low dose levels which are not associated with these changes, it will be clear that they are not necessary precursors of tumour development. Studies with other animal species and with other bone-seeking isotopes will also be of importance in assessing the significance of these proliferative lesions.

Finally, the " composite " lesions noted in the present paper must be considered. It is possible that each of them represents two or more originally separate tumours which have later become confluent. Alternatively, and this is the interesting possibility, the areas of highly cellular pleomorphic tissue may represent areas of more rapid growth, with consequent loss of tissue differentiation, in a pre-existing more highly differentiated tumour.

3. *Quantitative retention of ^{90}Sr in the skeleton*

It has already been pointed out that figures given in the tables for ^{90}Sr retention in the whole skeleton are approximate, since they are calculated from measurements on only part of the skeleton. The values obtained for ^{90}Sr in the tibia or femur, are all measured values and are therefore more reliable. They show that there is some variability among animals within the same group in ^{90}Sr retention measured per g. bone or per g. Ca. This variability may occur because of differences in ^{90}Sr intake due to secondary ingestion from coprophagy or because of variation in the daily calcium intake due to differences in appetite. The number of animals in the experiment is small so that it was not possible to determine whether the variability in ^{90}Sr retention was related in any way to the age or weight of the animal when feeding started, or to the terminal calcium content of the bone.

As would be expected, the retention of ^{90}Sr in the skeleton, expressed as a percentage of the amount fed, was greater in the rabbits that started feeding when 5–8 weeks old than in rabbits which started feeding when $3\frac{1}{2}$–4 years old. The retention of ^{90}Sr in young rabbits fed for 9 days, 31 days and 224–280 days was, on average, 42, 46 and 10 per cent respectively of that ingested.

It should be noted in a comparison of the figures in Table I that rabbits fed for periods up to 9 days received 11·8 μc, the 31 day rabbits 8·0 μc and the long term rabbits on average 8·5 μc daily. Allowance being made for these differences in daily intake, it can be seen that the rate of increase of ^{90}Sr per g. of Ca in the tibia is rapid up to 9 days, slightly slower from 9 till 31 days and much slower after 31 days. Under the present experimental conditions the quantity of ^{90}Sr per gram of calcium, averaged as it is throughout the bone, depends in a complicated way on many factors. The two most important are: (*a*) the ratio of non-active (i.e. bone formed before feeding started) to radioactive bone (i.e. bone formed after feeding started), and (*b*) the ratio of the ^{90}Sr to Ca in the blood. It is obvious that (*a*) has its greatest value at the start of ^{90}Sr feeding and decreases thereafter,

while (*b*) gradually decreases throughout the period of feeding. This decrease occurred because the amount of ^{90}Sr fed per day was approximately the same throughout the experiment while the Ca intake increased as the animal grew bigger. The rapid increase in the quantity, i.e. the amount of ^{90}Sr per gram of Ca, in the initial period of ^{90}Sr feeding is mainly due to the decrease in (*a*). Decrease in (*b*) would depress this quantity and would be particularly important in the latter stages of the experiment. Under ideal theoretical conditions, such as a constant ^{90}Sr/Ca ratio in the blood supply from the time of conception, the ^{90}Sr per gram of Ca in the bone would probably be constant with time.

The figures for ^{90}Sr content of the newborn rabbits and sucklings confirm previous observations, namely, that little ^{90}Sr passes from the mother's skeleton to the foetus but that considerable quantities of ^{90}Sr pass in the milk (Kidman, Tutt and Vaughan, 1951) probably coming from the ^{90}Sr of the mother's blood rather than from her bones. As would be expected ,^{90}Sr retention was much less in the bones of rabbits four years old when feeding started than in younger animals.

Attention has been drawn elsewhere (Holgate, Mole and Vaughan, 1958 ; Holgate, 1959) to the heavy uptake of ^{90}Sr in the teeth in both injected and fed animals, and it has been suggested that the high incidence of tumours in the mandible and maxilla in either animals fed or injected may be due to radiation from ^{90}Sr concentrated in the teeth rather than in the bones themselves. It is to be noted that all five weanlings fed for more than 200 days had multiple tumours in the mandibles and maxillae associated with the roots of the molar or incisor teeth. There is also an even, high uptake in the teeth of an old rabbit fed for a long period, which is in striking contrast to the slight irregular uptake in jaw-bone. This suggests that in older animals the ^{90}Sr content of the teeth may present a greater hazard than the bone uptake.

4. *The pattern of distribution of* ^{90}Sr

The present study, using autoradiographs of thin sections, has shown that in young rabbits fed ^{90}Sr the isotope is taken up in bone formed after the start of feeding (Fig. 24) and that within this bone the uptake is to some extent non-uniform. This unevenness is probably accounted for by two factors, (i) the decrease in the ^{90}Sr/Ca ratio of the diet in the course of feeding, mentioned above and (ii) the normal variation in calcium content of different parts of the bone. The variation in calcification in normal rabbit bone is not great and the size of the bone structures small so that in fact the uniformity of radiation dose-rate received by the bone is scarcely affected by this. The radiation dose-rate received by epiphyseal bone recently formed was found to be less than that received by bone formed soon after feeding started (Owen and Vaughan, 1959). This is due no doubt to the progressive decrease of the ^{90}Sr/Ca ratio in the diet.

The observations made here on the distribution of ^{90}Sr following daily feeding suggest that provided the ^{90}Sr/Ca content of the diet is constant the calcium of bone will be evenly labelled with ^{90}Sr in young rabbits.

In old rabbits the picture is different. In the bones of fully grown rabbits apposition occurs continuously but in a patchy way. When ^{90}Sr is fed there are rather more ^{90}Sr-containing patches than when ^{90}Sr is given as a single injection, since the circulating blood level of ^{90}Sr is maintained, but the pattern of uptake is the same following both methods of administration.

SUMMARY

1. ^{90}Sr has been fed daily to 24 rabbits of different ages for varying periods at the rate of either 8–11·8 μc or 25 μc per day. Rabbits fed 8–11·8 μc from the age of 5–8 weeks which survived for longer than 200 days developed multiple osteosarcomas at the growing ends of their long bones and in the bone surrounding maxillary and mandibular molar teeth. No skeletal lesions were seen in two rabbits over 3 years old when feeding started nor in sucklings born to rabbits fed ^{90}Sr.

2. Varying degrees of anaemia, leucopaenia and thrombocytopaenia occurred in rabbits aged 5–8 weeks when ^{90}Sr feeding began but not in the older age group. The marrow showed variable degrees of aplasia in younger rabits, though areas of active haemopoiesis could always be found.

3. The bone tumours were osteosarcomas which appeared to arise from the whole endosteal surface of the more rapidly growing ends of the long bones, including the epiphysis. Two of the tumours had conspicuous vascular spaces.

4. Patchy bone necrosis (i.e. empty lacunae and deficient staining) was widespread, but such bone damage was not invariably associated with demonstrable occlusion of blood vessels. Neither fibrosis adjacent to bone surfaces nor gross failure of resorption was conspicuous.

5. On prolonged feeding of weanling rabbits with 8–11·8 μc ^{90}Sr daily the rate of increase of ^{90}Sr per g. of calcium was at first rapid and then fell off with time. When the daily intake of ^{90}Sr was increased from 11·8 to 25 μc daily the retention of ^{90}Sr per g. of bone or per g. of calcium increased in approximately the same ratio.

6. The distribution of ^{90}Sr in bone formed after feeding started was slightly uneven in young rabbits where the bones were growing in length, there being less in the more recently formed bone. The reasons for this are discussed. The distribution in the bones of old rabbits was patchy, as in old rabbits receiving ^{90}Sr by injection.

We are indebted to Professor Martin Rushton and Surgeon Captain W. Holgate, R.N., for comments on the pathological changes seen in the teeth, and to Mrs. S. Groves, Miss J. Gardner, Miss D. Gross and Miss F. Schofield, for technical help.

REFERENCES

Hellner, H.—(1937) *Arch. klin. Chir.*, **189**, 706.
Holgate, W.—(1959) *Brit. dent. J.*, **107**, in the press.
Idem, Mole, R. H. and Vaughan, J.—(1958) *Nature, Lond.*, **182**, 1294.
Jee, W. S. S., Arnold, J. S. and Cochran, T. H.—(1957) *Anat. Rec.*, **127**, 424.
Jowsey, J.—(1955) *J. sci. Instrum.*, **32**, 159.
Kidman, B., Rayner, B., Tutt, M. and Vaughan, J.—(1952) *J. Path. Bact.*, **64**, 453.
Idem, Tutt, M. L. and Vaughan, J.—(1950) *Ibid.*, **62**, 209.—(1951) *Ibid.*, **63**, 253.
Kuzma, J. F. and Zander, G.—(1957*a*) *Arch. Path.*, **63**, 198.—(1957*b*) *Amer. J. Path.*, **33**, 607.
Lamerton, L. F. and Harriss, E. B.—(1954) *J. photogr. Sci.*, **2**, 135.
Lisco, H.—(1956) *In* Ciba Foundation Symposium on Bone Structure and Metabolism, London, p. 272.
Litvinov, N. N.—(1957) *Arkh. Pat.*, **19**, No. 1, p. 26.

MARTLAND, H. S.—(1931) *Amer. J. Cancer*, **15**, 2435.
OWEN, M., JOWSEY, J. AND VAUGHAN, J.—(1955) *J. Bone Jt. Surg.*, **37 B**, 324.
Idem, SISSONS, H. A. AND VAUGHAN, J.—(1957) *Brit. J. Cancer*, **11**, 229.
Idem, AND VAUGHAN, J.—(1959) *Ibid.*, **13**, 424.
PETROV, N. N., KROTKINA, N. A., VADOVA, A. V. AND POSTNIKOVA, Z. A.—(1952) 'Dynamics of the Origin and Development of Malignant Growths in Experiments on Monkeys.' Moscow (Press of the U.S.S.R., Academy of Medical Sciences).
ROSS, J. M.—(1936) *J. Path. Bact.*, **43**, 267.
SCHÜRCH, O., AND UELINGER, E.—(1935) *Arch. klin. Chir.*, **183**, 704.
SHELDON-PETERS, J., AND VAUGHAN, J.—(1956) *Brit. J. exp. Path.*, **37**, 553.
SINCLAIR, W. K., ABBATT, J. D., FARRAN, H. E. A., HARRISS, E. B., AND LAMERTON, L. F.—(1956) *Brit. J. Radiol.*, **29**, 36.
VAUGHAN, J. AND OWEN, M.—(1959) *Lab. Invest.*, **8**, 181.

RADIATION DOSE AND ITS RELATION TO DAMAGE IN THE RABBIT TIBIA FOLLOWING A SINGLE INJECTION AND DAILY FEEDING OF ^{90}Sr

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STRONTIUM is a bone-seeking isotope which behaves like calcium in that it is laid down in bone in sites of active growth. Consequently, radioactive strontium when taken up in the body will become fixed in bone and in sufficient quantities it is known to do extensive damage, ultimately producing bone tumours. A considerable amount of data has been collected on the production of tumours in relation to the initial injected dose of radioactive strontium, measured in μc/kg. of body weight (Brues, 1949; Finkel, Lisco and Brues, 1955) and recently a comparison has been made in the same terms of the potency of different bone-seeking isotopes for bone tumour production (Finkel, 1956).

So far, however, little has been done on determining the actual radiation dose to the bone or particular parts of the bone and comparing this with the radiation damage. In previous work a study has been made of the terminal radiation damage in the upper half of the rabbit tibia, (*a*) following a single intravenous injection of ^{90}Sr (Owen, Sissons and Vaughan, 1957) and (*b*) following a period of daily feeding of pellets containing ^{90}Sr (Downie, Macpherson, Ramsden, Sissons and Vaughan, 1959). There were distinctive differences between the radiation damage found in the two series. The purpose of the present paper is to attempt to determine the pattern of dose-rate and the accumulated radiation dose received by different parts of the rabbit tibia following a single injection or daily feeding of ^{90}Sr, and to compare this with the pattern of bone damage and production of bone tumours in the terminal cases of (*a*) and (*b*) above. The relationship of radiation dose to early changes in the bone and marrow will form the subject of a later paper.

EXPERIMENTAL TECHNIQUES

Dutch rabbits of the same stock as in previous experiments were used (Tutt, Kidman, Rayner and Vaughan, 1952). Weanling rabbits, aged 5–8 weeks, were either given a single intravenous injection of 100 or 600 μc of ^{90}Sr/kg. body weight or started daily feeding of pellets containing approximately 8·5 μc of ^{90}Sr. They died or were killed at varying time intervals up to 9 months later. The methods of injecting and feeding ^{90}Sr are described in detail in the previous publications. In the case of the animals injected, the dosimetry measurements were made on a different series of animals to those previously described. In the case of the animals fed ^{90}Sr, the same animals were used.

In general, the left tibia was used for either estimation of ^{90}Sr retention or kept for histological observations. The right tibia was used both for radiation

dosimetry measurements and for the preparation of microradiographs and autoradiographs. The histological techniques and the preparation of microradiographs and autoradiographs have been described in the previous two papers. The method of measuring radiation dose-rates by determining the density of autoradiographs of thick sections and comparing it with that from a known standard is described in detail in a recent paper (Owen and Vaughan, 1959).

RESULTS

In the two previous papers (Owen, Sissons and Vaughan, 1957; Downie, Macpherson, Ramsden, Sissons and Vaughan, 1959) the terminal radiation damage following a single injection and a period of daily feeding of ^{90}Sr, has been described in detail. In the series of injected rabbits, the animals were given 500–1000 μc of ^{90}Sr per kg. body weight at the age of 5–8 weeks, in the series of rabbits fed ^{90}Sr daily, the animals were fed a daily pellet containing about 8·5 μc of ^{90}Sr from the age of 5–8 weeks. In both series the animals died from 6 to 8½ months later with multiple bone tumours and blood dyscrasies. The terminal burdens of ^{90}Sr in the skeletons at the time of death was of the same order of magnitude, approximately 100 μc and 180 μc in the two series respectively.

The main results of the present paper are measurements of the dose-rate and the accumulated dose received in the upper half of the tibia, and an attempt to correlate them with the radiation damage observed. The pattern of uptake and retention of ^{90}Sr and of radiation damage in this portion of bone has already been described in the papers mentioned above. For the sake of completeness, however, a brief summary of the results obtained in these papers is given below.

Uptake and Retention of ^{90}Sr

Fig. 1 shows the main regions of retained ^{90}Sr in a longitudinal section through the middle of the posterior wall and the lateral-medial corner of the proximal half of the tibia of a rabbit aged 7 months, (*a*) given a single injection and (*b*) started daily feeding of ^{90}Sr, at the age of 5–8 weeks. The regions of bone which existed before injection of ^{90}Sr or before ^{90}Sr feeding started are illustrated in (*c*). The scale at the side is in millimetres and the numbers correspond to the levels referred to in the text. The figure shows a simplified picture of the main regions of ^{90}Sr retention only. There is no indication of the relative concentrations of ^{90}Sr in the different regions. ^{90}Sr is also taken up diffusely at low concentration throughout bone and this is not indicated in the figure. Uptake in remodelling Haversian canals within the bone formed before injection or start of ^{90}Sr feeding, and secondary uptake in bone formed after injection from level 30 upwards are not shown.

After injection the ^{90}Sr is immediately taken up in three main regions of growth, in the calcifying cartilage and trabecular bone beneath the epiphyseal plate and in the periosteal and endosteal bone of the shaft and metaphysis. The ^{90}Sr retained in the posterior wall at about level 28 (Fig. 1*a*) is mainly due to retention of ^{90}Sr in unresorbed cartilage remnants and the bone covering their surfaces. The amount of ^{90}Sr retained in this region depends on the injected dose as is seen below, a greater proportion being retained at the higher dose level. This region is approximately the level of the plate at the time of injection, i.e. at the age of 5–8 weeks.

In the rabbits which were started feeding ^{90}Sr at the age of 5–8 weeks ^{90}Sr is taken up throughout the bone formed after this period. Above level 28 (Fig. 1*b*) it is laid down in the process of epiphyseal growth and below this level it is taken up in broad bands on the endosteal and periosteal surface in the process of transverse growth.

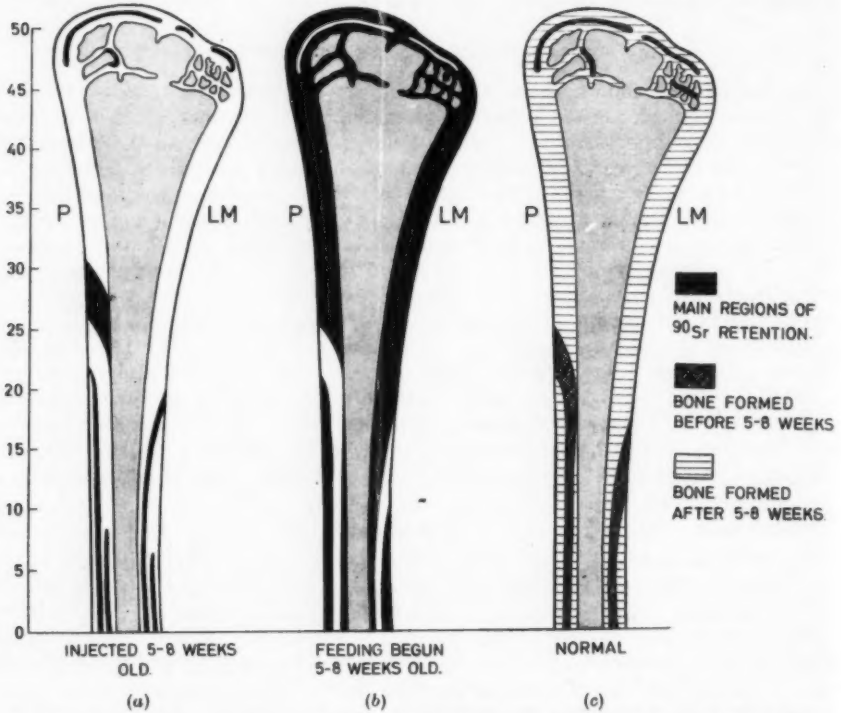


FIG. 1.—Diagrammatic representation of a longitudinal section through the middle of the posterior wall (P) and the lateral-medial (LM) corner of the proximal half of the tibia of a rabbit aged 7 months. Illustrates regions of ^{90}Sr retained, (*a*) following a single injection at the age of 5–8 weeks, (*b*) after daily feeding of ^{90}Sr from the age of 5–8 weeks, (*c*) shows the regions of bone which existed before the age of 5–8 weeks. Scale at side is in millimetres, numbers correspond to levels referred to in the text. o is approximately the middle of the bone.

Dose-rate Measurements in Bone

Dose-rate patterns in a longitudinal direction in the posterior wall in the proximal end of the tibia, and in a transverse direction in the middle of the shaft have been measured at different times after injection or start of ^{90}Sr feeding.

Single injection

The 600 μc/kg. dose was chosen as being comparable with the range of injected doses (500–1000 μc/kg.) given in the previous experiments. At this dose level

there is severe radiation damage, the dose-rate pattern was therefore also studied at 100 μc/kg., at which level there appears to be no gross interference with bone growth (Owen, Jowsey and Vaughan, 1955). Briefly, following a dose of 100 μc/kg. the maximum dose-rate up to 30 days after injection was being received by the tissues beneath the epiphyseal plate at the time of injection. (Owen and Vaughan, 1959). By six months after injection most of the ^{90}Sr had been resorbed from this region and the maximum dose-rate was being received in the middle of the bands of ^{90}Sr taken up on the periosteal and endosteal surfaces.

In the case of the rabbits given 600 μc/kg. there is, however, gross failure of resorption of the cartilage and bone which took up ^{90}Sr at the level of the epiphyseal plate at the time of injection and in the majority of cases, the maximum dose-

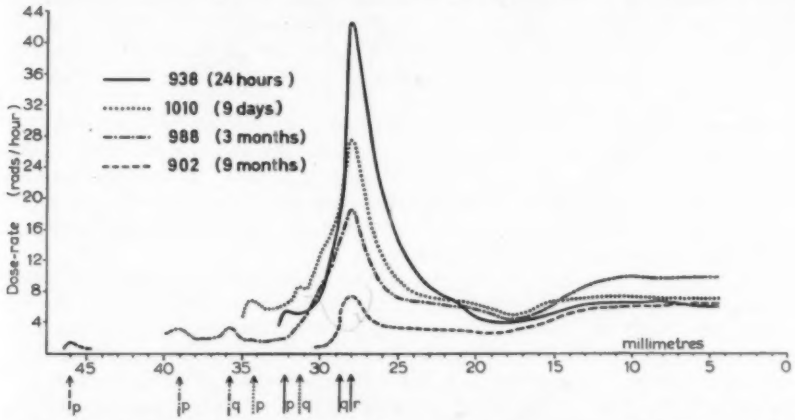


FIG. 3.—Variation in dose-rate at different times after injection measured along the middle of the posterior wall of proximal end of tibia (see arrow Fig. 2) of rabbits given single injection 600 μc/kg. at age of 5–8 weeks. p, q and r correspond respectively to upper and lower bone of epiphysis and bone beneath epiphyseal plate at time of injection (Fig. 2).

rate at all times after injection was being received by this region. Fig. 2*a* shows an autoradiograph of the longitudinal proximal half of the tibia of a rabbit given 600 μc/kg. and killed 30 days later. This gives a picture of the dose-rate distribution in the middle of the posterior wall and the lateral-medial corner. Comparison with that from a rabbit given 100 μc/kg. (Fig. 2*b*) illustrates the greater retention of ^{90}Sr at the higher injected dose level. In Fig. 2 the positions of the deposition of ^{90}Sr in the upper and lower bony surfaces of the epiphysis and in the calcifying tissues below the epiphyseal plate at the time of injection are denoted by p, q and r respectively. The dose-rate was measured along the middle of the posterior wall (i.e. in the direction of the arrow, Fig. 2). A selection of curves for the rabbits given 600 μc/kg. is shown in Fig. 3. The curves are similar to those for rabbits given 100 μc/kg. (Owen and Vaughan, 1959) except that the maximum dose-rates fall off less rapidly with time after injection and a peak persists at the level of the epiphyseal plate at the time of injection.

In the metaphysis and shaft ^{90}Sr is taken up in bands on the periosteal and endosteal surfaces. The major proportion of these bands is retained 6 months

later. In Fig. 4 an autoradiograph of a thick section shows the dose-rate distribution in a transverse plane due to uptake of ^{90}Sr on the periosteal surface of the posterior wall and on the endosteal surfaces of the lateral and medial walls, at about level 13 (Fig. 1).

Results for the rabbits given a single injection are collected together in Table I. The dose-rates being received in the middle of the upper bone of the epiphysis p, and in the calcifying tissues r, beneath the plate at the time of injection, are given in column 5. The maximum dose-rates measured in the midle of the bands of periosteal and endosteal uptake and in the middle of the marrow in the shaft are given in column 6. The amount of ^{90}Sr injected is proportional to the weight of the animal, and for a better comparison of the results the dose-rates should be corrected assuming all the rabbits were the same weight when injected. This has been done for the dose-rates in the region of the epiphyseal plate at the time of injection, i.e. r, column 5, Table I, and the result is plotted in Fig. 5 in curves A and B. It is clear that up to 24 hours after injection the dose-rates in the " 600 μc " series are approximately six times those in the " 100 μc " series. At longer periods after injection, however, there is evidence of relatively higher dose-rates in the " 600 μc " series. In both series there is considerable variation between rabbits at the same time interval after injection. This variation among rabbits constitutes the largest error in estimating the accumulated dose received by any region. It should be mentioned, however, that the results for litter mates showed slightly less variation than others.

The maximum dose-rates measured in the middle of the bands of uptake on the periosteal and endosteal surfaces and in the marrow in the middle of the shaft, are given in column 6. There was no evidence that the dose-rates in the middle of these bands of uptake fell off with time after injection, though there was a considerable spread in the results at any time interval. The dose-rates to the periosteal and endosteal surfaces fall off with time, however, as the bands become buried by the apposition of new bone on these surfaces. Note the position of the bone surfaces with regard to the middle of the bands, i.e. the region of maximum dose-rate (Fig. 4*b*).

EXPLANATION OF PLATES.

FIG. 2.—Autoradiographs of longitudinal proximal halves of tibia illustrating dose-rate distribution in a plane through the middle of the posterior wall (arrowed) and the lateral-medial corner, 30 days after a single injection of ^{90}Sr, (*a*) 600 μc/kg., (*b*) 100 μc/kg. p, q and r correspond respectively to upper and lower bone of epiphysis and bone immediately beneath epiphyseal plate at time of injection. Levels on left same as Fig. 1.

FIG. 4.—(*a*) Autoradigraph of thick transverse section illustrating dose-rate distribution in the middle of the shaft, due to uptake on the periosteal surface of the posterior wall P, and the endosteal surface of the lateral-medial corner LM, in rabbit nine months later. (*b*) Graph of variation in dose-rate along line in (*a*), the vertical lines indicate the positions of the periosteal and endosteal surfaces.

FIG. 6.—Autoradiographs of longitudinal proximal halves of tibia illustrating dose-rate distribution in a plane through the middle of the posterior wall (arrowed) and the lateral-medial corner, (*a*) 30 days, (*b*) 263 days after daily feeding of ^{90}Sr from the age of 5–8 weeks. Levels on left same as Fig. 1.

FIG. 8.—(*a*) Autoradiograph of thick transverse section illustrating dose-rate distribution in the middle of the shaft, due to ^{90}Sr uptake on the periosteal surface of the posterior wall P, and the endosteal surface of the lateral-medial corner LM, in rabbit fed ^{90}Sr daily from the age of 5–8 weeks, died 263 days later. (*b*) Graph of variation in dose-rate along line in (*a*), the vertical lines indicate the positions of the periosteal and endosteal surfaces.

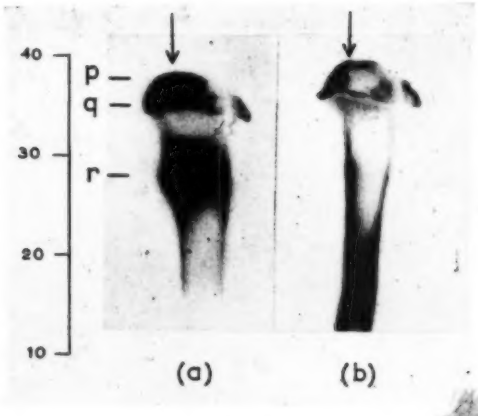
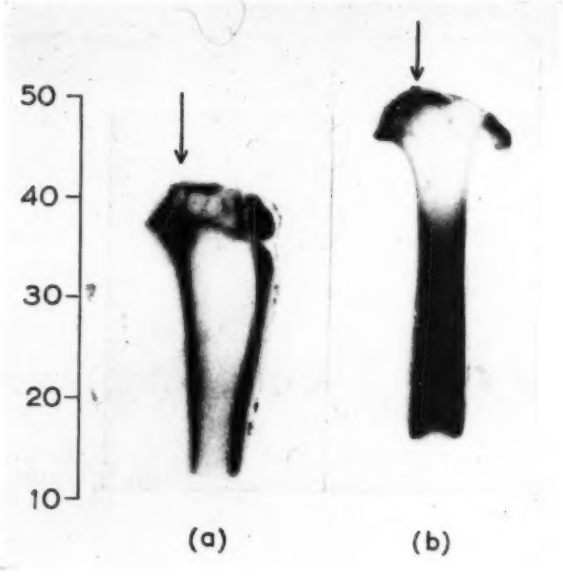


Fig. 2.



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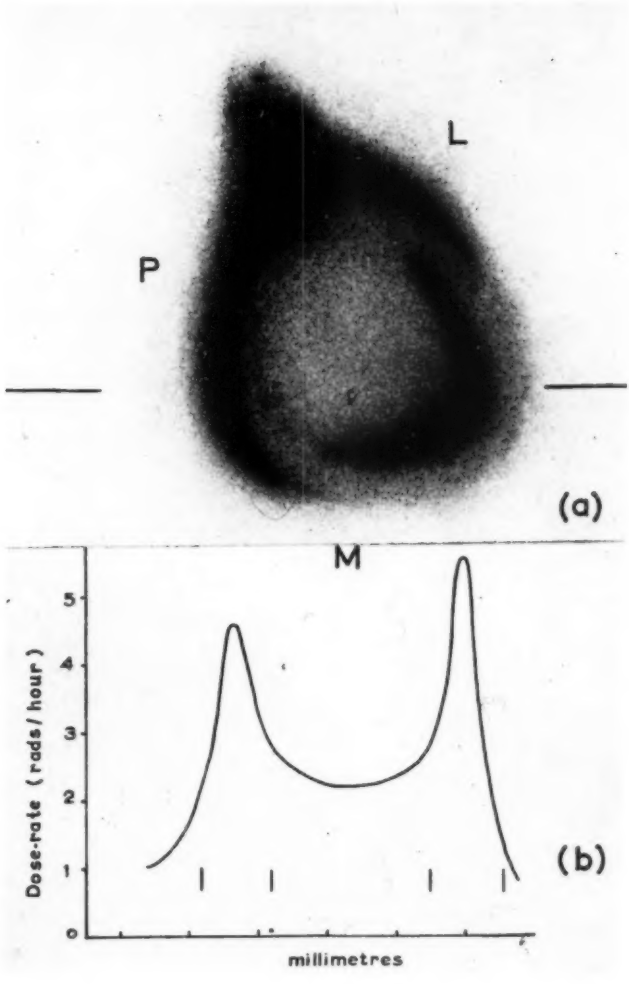

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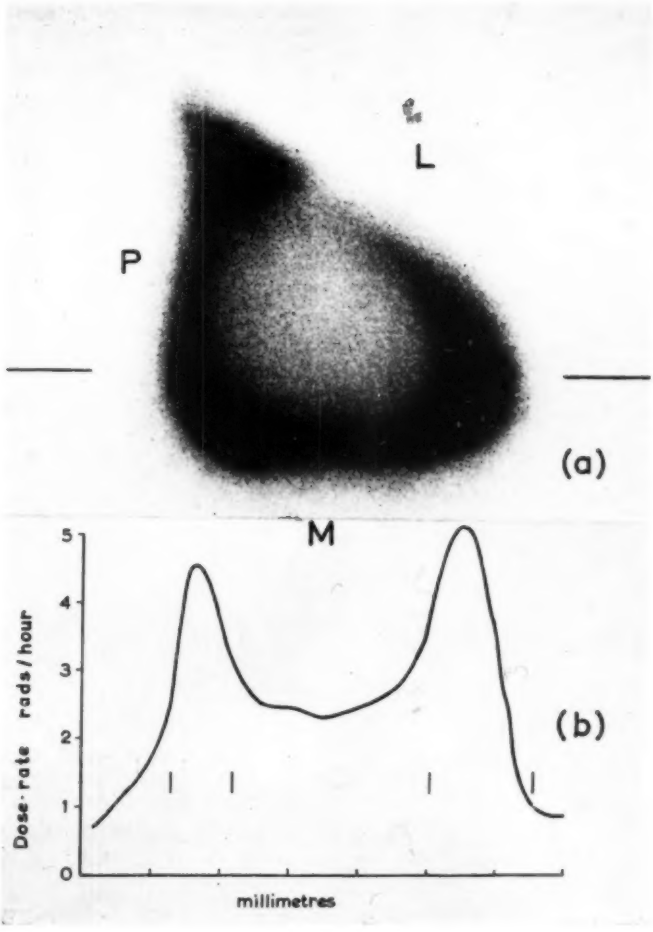

Fig. 8.

jected dose

600 μc of ^{89}Sr/kg. body weight.

100 μc of ^{89}Sr/kg. body weight.

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TABLE I.—*Measured Dose-rates in the Rabbit Tibia Following a Single Intravenous Injection of ^{90}Sr at the Age of 5–8 Weeks*

Injected dose	Rabbit number (1)	Weight (g.) When injected (2)	Weight (g.) When killed (2)	Age when injected (days) (3)	Time killed after injection (4)	Measured dose-rate (rads/hour) *p (5)	†r (5)	Middle of periosteal uptake (6)	Middle of endosteal uptake (6)	Middle of marrow in shaft
600 μc of ^{90}Sr/kg. body weight.	958	580	580	40	1 hour	9·7	55	3·8	—	2·0
	954	620	620	40	8 hours	6·2	43	3·2	—	1·9
	991	570	560	55	8 ,,	6·6	35	6·3	5·4	2·5
	938	670	670	42	24 ,,	5·4	42	5·6	—	2·3
	966	500	480	46	24 ,,	—	26	4·7	7·8	1·8
	989	610	590	55	24 ,,	6·3	30	9·8	6·7	3·2
	953	640	710	41	3 days	4·9	19·6	3·5	—	—
	960	530	530	45	3 ,,	3·3	12·7	—	—	—
	1009	640	650	45	3 ,,	7·2	33	5·2	—	2·5
	939	660	690	42	9 ,,	3·4	12·3	6·4	—	1·8
	965	580	660	46	9 ,,	—	12·0	4·3	8·8	1·6
	1010	600	650	45	9 ,,	6·6	27	6·8	7·4	2·8
	1079	590	740	46	9 ,,	5·9	14·5	5·8	—	1·9
	1080	590	660	46	16 ,,	3·9	7·8	4·6	—	1·5
	937	660	820	42	30 ,,	4·4	20	7·2	—	3·0
	1012	590	470	45	30 ,,	4·6	21·8	6·6	—	2·8
	988	690	980	55	3 months	3·0	15·2	11·1	9·1	4·3
	990	690	730	55	4½ ,, D	3·4	18·3	10·8	10·4	2·4
	1077	620	1270	46	6 ,,	3·1	4·8	—	—	—
	902	840	1320	50	9 ,,	1·5	7	5·3	—	2·0
100 μc of ^{90}Sr/kg. body weight.	969	640	640	48	1 hour	0·8	5·7	0·61	—	0·28
	993	670	670	41	8 hours	1·6	8·6	1·22	—	0·47
	983	620	620	42	8 ,,	1·6	6·4	0·73	—	0·27
	1082	720	720	48	8 ,,	—	5·4	0·84	—	0·35
	996	540	540	49	24 ,,	0·9	6·2	0·84	—	0·34
	934	580	580	40	24 ,,	1·3	5·0	0·80	—	0·33
	1083	710	720	48	24 ,,	—	4·4	0·80	—	0·33
	999	510	520	49	3 days	0·86	4·1	1·1	0·98	0·34
	1060	580	550	47	3 ,,	0·83	2·2	0·86	—	0·26
	997	480	550	49	9 ,,	0·61	2·0	0·96	0·75	0·28
	1061	590	680	47	9 ,,	0·61	1·0	1·0	0·92	0·39
	998	540	750	49	30 ,,	0·48	1·1	1·1	0·96	0·33
	952	740	1220	41	30 ,,	0·45	0·52	—	0·94	0·32
	1062	600	980	47	30 ,,	0·48	0·75	—	—	—
	1000	570	1340	49	3 months	0·5	0·47	1·1	0·90	0·38
	992	470	1340	55	6 ,,	0·44	0·14	1·0	0·92	0·40
	970	670	2250	48	6 ,,	0·26	0·24	—	0·98	0·37

* p is the middle of the upper bone surface of the epiphysis.
† r is the middle of the region of maximum uptake of ^{90}Sr at the level of the epiphyseal plate at the time of section.
D = died.
Where no figure is given the measurement was not made.

Pellet fed

Fig. 6*a* and *b* are longitudinal autoradiographs of the bone halves of the proximal end of the tibia of rabbit 973 killed 30 days and 932 which died 263 days after start of daily feeding of ^{90}Sr pellets. They illustrate the distribution of dose-rate at the time of death in the middle of the posterior wall and the lateral-medial corner. Curves for the variation in dose-rate along the middle of the posterior wall at various times after start of feeding are shown in Fig. 7. Level 28 is the approximate position of the epiphyseal plate at the time feeding started. The two main regions of uptake are bone formed below the epiphyseal plate in the process of growth in length, and bone formed on the periosteal and endosteal surfaces of the shaft and metaphysis (Fig. 1). At short times after start of feeding

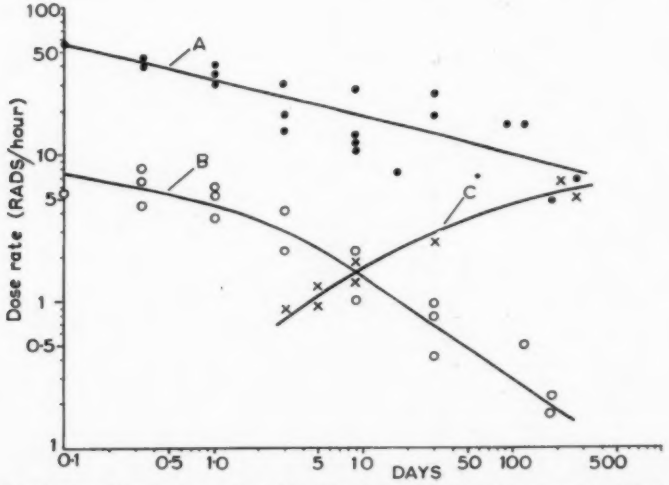

FIG. 5.—Graph of maximum dose-rate at different times after injection or start of ^{90}Sr feeding. Rabbits injected with 600 μc of ^{90}Sr/kg., curve A, 100 μc of ^{90}Sr/kg., curve B, at the age of 5–8 weeks, each figure has been corrected assuming the rabbits weigh 600 g. when injected. Curve C is for rabbits fed 8·5 μc of ^{90}Sr daily from the age of 5 to 8 weeks.

the dose-rate is a maximum in the bone being formed below the epiphyseal plate. At 30 days the dose-rate has reached an approximately constant value throughout the bone length. After this the dose-rate in the shaft and lower part of the metaphysis continues to increase and reaches a maximum of about 5·5 rads/hour 9 months after start of feeding. In the upper part of the metaphysis the dose-rate is less, e.g. in rabbit 932 the dose-rate at level 44 (Fig. 1), is about 1·2 rads/hour. This bone has been formed relatively recently and the fall off in dose-rate is probably largely due to the fact that the $^{90}Sr/Ca$ level in the blood decreased as the animal grew older, (Downie Macpherson, Ramsden, Sissons and Vaughan, 1959). The dose-rate distribution at about level 13 in the shaft is shown in Fig. 8. It is to be noted that in this case the bone surfaces received a greater fraction of the maximum dose-rate than in the injected animals (compare Fig. 4*b* and 8*b*).

Results for the pellet fed animals are given in Table II. The measured maximum dose-rates in the posterior wall at the level of the epiphyseal plate at the time feeding started are shown in column 4 and in curve C, Fig. 5. The maximum dose-rate in the middle of the bands of uptake on the periosteal and endosteal surfaces is given in column 5. About 30 days after feeding started this had reached the same value as that in bone formed below the epiphyseal plate. The dose-rate in the middle of the marrow cavity in the shaft is shown in column 6. Two rabbits only were in most cases measured at each time interval, the agreement between them was reasonably good. The terminal maximum dose-rates in the 600 μc rabbits and the ^{90}Sr fed rabbits were approximately the same, as can be seen from curves A and C, Fig. 5.

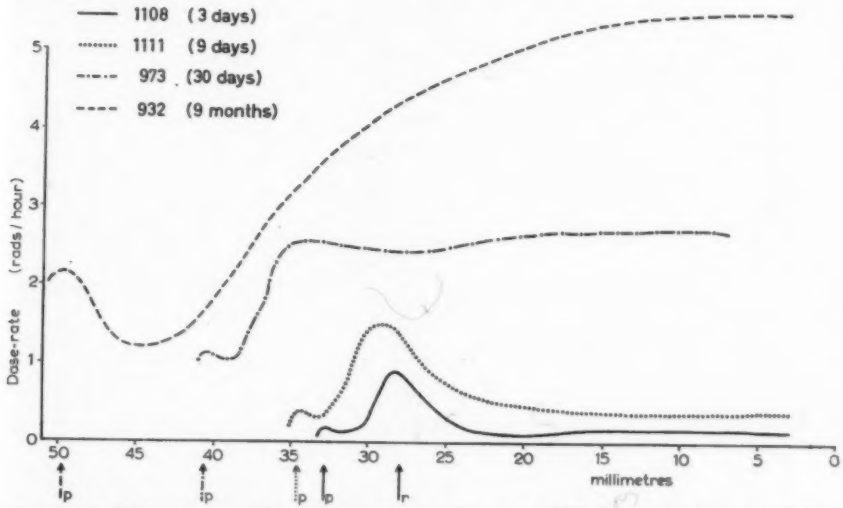

FIG. 7.—Variation in dose-rate at different times after start of ^{90}Sr feeding at the age of 5–8 weeks, measured along the posterior wall of proximal end of tibia (arrow, Fig. 6). p and r correspond respectively to upper bone of epiphysis and bone immediately beneath epiphyseal plate at time feeding started.

Dose-rate Measurements in the Marrow

In both the rabbits given a single injection and those fed ^{90}Sr daily the dose-rate in the middle of the marrow cavity was generally about half the dose-rate received in the middle of the posterior wall of the bone at the same level. The dose-rate across the marrow was fairly constant (Fig. 4*b* and 8*b*), marrow adjacent to bone never receiving more than about 1½ times that in the middle of the marrow. Typical dose-rates in the middle of the marrow in the shaft in the two series are given in columns 6 of the tables.

The dose-rate to the marrow follows the same pattern with time as the dose-rate to bone (Fig. 3 and 7). In the case of the injected animals the marrow tissues in the region of the epiphyseal plate at the time of injection, i.e. level 28, receive an initial dose-rate equivalent to that received by tissues r (Fig. 3). This falls off with time after injection though there is nearly always a peak at this level,

due to failure of resorption of the ^{90}Sr in the nearby bone. In contrast with the rabbits given a single injection, in the pellet fed animals the dose-rate builds up gradually with time after start of feeding.

TABLE II.—*Measured Dose-rates in the Rabbit Tibia Following Daily Feeding of ^{90}Sr from the Age of 5–8 Weeks*

Rabbit (1)	Amount of ^{90}Sr per pellet ($\mu c.$) (2)	Days on pellets (3)	Measured dose-rate (rads/hour) corrected to 8·5 $\mu c.$/pellet*		
			At the level at which the epiphyseal plate was at time feeding started (4)	Middle of periosteal or endosteal uptake (5)	Middle of marrow in shaft (6)
1108	11·8	3	0·89	0·13	0·06
1110	11·8	5	0·92	0·15	0·05
1112	11·8	5	1·23	0·19	0·06
1111	11·8	9	1·5	0·37	0·15
1109	11·8	9	1·8	0·26	0·09
973	8·5	30	2·6	2·65	1·0
930	8·5	210	5·0	6·0	2·3
932	8·5	263	4·3	5·5	2·2

* The dose-rates for the rabbits given 11·8 $\mu c.$/pellet have been proportionately reduced to make them comparable with the rabbits given 8·5 $\mu c.$/pellet.

Radiation Damage and Radiation Dose

Bone

In the rabbits given a single injection examination of their tibiae showed gross damage, consisting mainly of unresorbed cartilage remnants with abnormal bone on their surfaces in the posterior wall at the level of the plate at the time of injection, about level 28 (Fig. 1). This region of gross failure of resorption and abnormal bone extended usually from about level 25 to 31, about 6 mm. of the bone length. The middle of the region was that of maximum uptake and retention of the ^{90}Sr in the calcifying cartilage just below the plate at the time of injection. The tumours in the tibiae occurred without exception towards the ends of the bones, in two cases out of the 6 rabbits studied they were small enough so that some attempt could be made at estimating their site of origin and it was found from examination of serial sections to be this region of maximum damage. Above the region of maximum damage the bone of the metaphysis had been formed after injection, it was abnormal but the degree of abnormality decreased as the distance from the level of initial uptake of ^{90}Sr increased. Below level 28, in the shaft and metaphysis, areas of dead bone were found associated with the bands of periosteal and endosteal uptake. The tibiae were also shorter and thinner than controls.

In the rabbits fed ^{90}Sr daily from the age of 5–8 weeks there was no significant failure of resorption of cartilage remnants but in many cases gross tumour extended throughout the ends of the tibiae and other long bones. In a few cases, where the tumour itself was small it could be seen that abnormal bone was present along

most of the endosteal bone surfaces, particularly on the posterior and medial walls. This widespread damage made it difficult to determine the exact site of tumour origin. There was also scattered injury throughout cortical bone which took the form of diffuse but patchy loss of osteocytes, sometimes, though not invariably associated with vascular injury. Such areas of injury were found particularly near bands of uptake in periosteal bone. In this case the tibiae were not noticeably shorter or thinner than the controls.

An attempt will be made, using the results in the present paper, to determine the accumulated dose received by different regions of the tibiae of the rabbits in the two series and to correlate this with the damage observed.

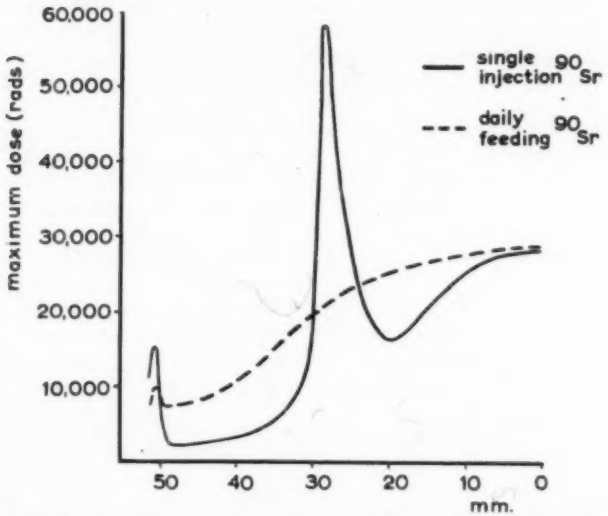

FIG. 9.—Variation in the maximum accumulated dose received at different levels in the posterior wall of the upper half of the rabbit tibia 200 days after a single injection of 600 μc of ^{90}Sr/kg. at the age of 5–8 weeks, 250 days after daily feeding of 8·5 μc of ^{90}Sr from the age of 5–8 weeks. Scale in mm. is same as in Fig. 1. o is approximately the middle of the bone, the level of the epiphyseal plate at the age of 5–8 weeks is approximately 28.

Accumulated doses received.—Using the curves for the variation in dose-rate along the bone, a selection of which are shown in Fig. 3 and 7, an estimate of the maximum accumulated dose received at different levels has been made both for rabbits given a single injection of 600 μc/kg. and rabbits fed 8·5 μc of ^{90}Sr daily. These are shown in Fig. 9. The doses are calculated for 200 days after injection, curve A, and 250 days after start of feeding, curve B. These were the average times of death in the two experiments. The variation in dose-rate among different rabbits measured at the same time interval, as mentioned earlier, means that the estimate of the accumulated dose received by any region is subject to a considerable range of error. There are, nevertheless, certain distinct differences between the curves in Fig. 9 which are of interest.

The portion of bone receiving 80 per cent or more of the maximum value of

the accumulated dose, is much less in the injected animals than in those fed ^{90}Sr. In the case of the injected animals it is about 1 mm. of bone length at level 28, in the case of the rabbits fed ^{90}Sr the region extended along a considerable length of the tibia examined, about 3 cm. from level 0 to 30 (Fig. 9). In the injected animals the maximum dose was received by a small region of trabecular bone including the marrow spaces and osteogenic connective tissues covering the bone surfaces. It will be noticed that the maximum dose in this case is much greater than in the case of the fed animals. From histological observations however (Owen, Sissons and Vaughan, 1957) it was observed that the tissues which had received the maximum dose were generally dead and it is more likely that the tumour arose from nearby tissue which had received a dose less than the maximum. In the case of the animals fed ^{90}Sr, the maximum dose from level 0 to 30 was received in the middle of the bands of ^{90}Sr uptake on the periosteal and endosteal surfaces, and there were many dead osteocytes associated with these areas.

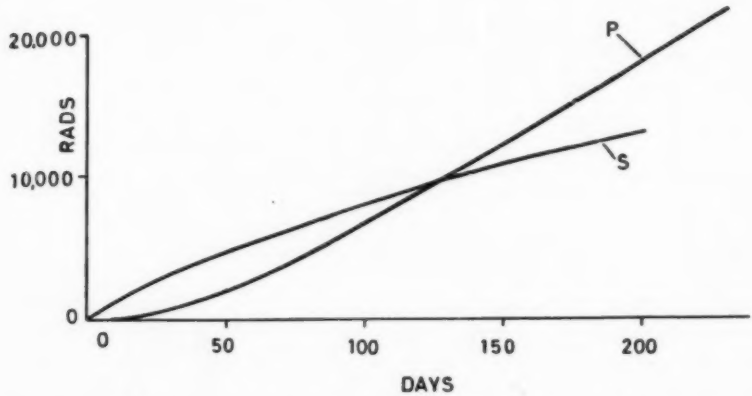

FIG. 10.—Graph of the accumulated dose received by the endosteal surface at different times, after a single injection of 600 μc of ^{90}Sr/kg., curve S, and after start of feeding of 8·5 μc of ^{90}Sr/kg., curve P, at the age of 5 to 8 weeks.

The regions of maximum damage and the probable site of tumour origin were, however, the endosteal surfaces, which in fact received a dose about 20 per cent less than the maximum dose. It will be noted at this point that in the shaft from level 0 to 5 (Fig. 9) the maximum dose in both series of animals is about the same; the dose received by the endosteal surfaces, however, which are the sensitive tissues, is much less in the injected animals as is illustrated below.

In the animals given a single injection of 600 μc of ^{90}Sr/kg. there was no sign of abnormal bone on the endosteal surfaces of the shaft similar to that seen in the fed animals. It is difficult to estimate the accumulated dose received by the bone surface since it depends on the rate at which ^{90}Sr becomes buried by new bone and this varies with the bone level. As an example, at level 13 where the dose-rate distribution in a transverse plane is as shown in Fig. 4 and 8 the accumulated dose received by the endosteal surface at the lateral medial corner at different

times after injection or start of ^{90}Sr feeding, has been calculated and is illustrated in Fig. 10. In the case of the fed animals the accumulated dose received by the endosteal surface over the period of the experiment is about 22,000 rads, in the case of the injected animals it is less, about 13,000 rads.

On the other hand, in the injected rabbits, the shafts were in general only about $\frac{3}{4}$ the diameter of the controls indicating at some stage inhibition of transverse growth, whereas in the rabbits fed ^{90}Sr daily, the bones were of normal thickness. This is probably related to the dose-rate pattern which is very different in the two cases. In the case of the fed animals the surfaces receive an initial dose-rate of only 0·13 rads/hour which increases gradually to about 4 rads/hour at 6 months. In the case of the injected rabbits it falls from an initial high dose-rate (it is approximately half the maximum in 30 days) as the ^{90}Sr deposit becomes buried by new bone. This initial high dose-rate of about 6 rads/hour to the young growing cells of the periosteal and endosteal surfaces at the age of 5–8 weeks may account for the inhibition of transverse growth in the case of the injected animals.

A further point of interest is the fact that no abnormal bone similar to that on the endosteal surfaces was seen on the periosteal surfaces of the pellet fed animals. Because of the cylindrical shape of the bone itself, the periosteal surfaces on the whole received a somewhat smaller dose than the endosteal surfaces though this is probably not sufficient to account for the differences in damage. Other differences between the two surfaces must also be important, for instance differences in oxygen supply.

Some unpublished observations of the authors indicate that the shortening of the tibiae in the injected rabbits is due mainly to the initial high dose-rate received by the sensitive growing tissues of the epiphyseal plate. It is likely also that the gross failure of resorption and resulting damage in this region is mainly due to this high dose-rate.

Marrow

In the injected animals there were no areas of active haemopoiesis, the marrow showed a complete gelatinous degeneration associated with profound anaemia and, with one exception, leucopaenia. In the animals fed ^{90}Sr there was considerable aplasia of the marrow but areas of active haemopoiesis were always found. Some small areas of mucoid degeneration were found in only one animal. There was no significant difference in the blood picture in the two groups, but the number of animals in both cases was small.

It is difficult to quantitate the marrow damage in the two series in any way. It can only be said that the damage was more severe in the case of the injected animals. The accumulated dose to the middle of the marrow in both series has about half the value of that received in the bone wall and follows the same pattern along the bone length (Fig. 9). With regard to the more severe damage in the case of the injected animals again the different dose-rate pattern may be significant. In the case of the injected animals a limited region of sensitive bone and marrow tissues just below the epiphyseal plate at the time of injection receive an initial very heavy dose-rate which then falls off with time after injection. In the case of the rabbits fed ^{90}Sr the dose-rate builds up gradually from zero with time after start of feeding.

Calculation of the Maximum Value of the Accumulated Dose from the Terminal Burden of ^{90}Sr

Often the only available information is the burden of ^{90}Sr in the bone. It was considered of interest, therefore, to compare the estimate of the maximum value of the accumulated dose made from the present measurements, with that calculated using the terminal burden of ^{90}Sr in the tibia and the non-uniformity factor, i.e. the ratio of the maximum dose-rate to the average dose-rate.

The terminal burden of ^{90}Sr in the tibia was known from radio-chemical measurements for three of the rabbits, 902, 930 and 932. The average dose-rates in the tibia were calculated from this, assuming uniform distribution of the ^{90}Sr and taking into account the limited dimensions of the bone, and the non-uniformity factors were taken as 6·3 and 2 for the injected and pellet-fed rabbits respectively (Owen and Vaughan, 1959). The maximum value of the accumulated dose calculated using these quantities is given by—

Time × average radiation dose-rate × non-uniformity factor. Time is the period for which the strontium remains in the bone. Using these figures a comparison of the calculated with the measured value of the maximum accumulated dose is made in Table III.

TABLE III.—*Maximum Value of Accumulated Dose*

	Injected	Pellet Fed.	
Rabbit number	902	930	932
Calculated	40,824	40,320	27,216
Measured	68,600	22,600	26,673

In the above cases it can be seen that the maximum accumulated dose calculated from the ^{90}Sr burden in the bone may differ from the measured value by a factor of 2. It is not possible to say whether this approaches an outside limit for the discrepancy since we are conscious of the scanty nature of the results particularly for the non-uniformity factors, which, furthermore, have been measured only for the tibia. In the injected animals the value was obtained from only one rabbit and in the fed animals it is an average of 2 rabbits. However for a variety of young and old rabbits non-uniformity factors measured in the tibia did not have a value greater than 7. What is important to remember though, is that the accumulated dose gives no indication of the different dose-rates to which the tissues may have been subjected throughout the period of exposure to the isotope. In particular, in the injected rabbits the maximum dose-rates immediately after injection were of the order of 7 times the terminal maximum dose-rate at 9 months after injection.

DISCUSSION

The chief difficulty in determining the radiation dose in the present experiments occurred because of the variability of the rabbits themselves. For the same time interval rabbits received a wide range of dose-rates resulting from differences in uptake and retention of ^{90}Sr. The estimate of the accumulated dose received by any site is thus subject to considerable uncertainty. Furthermore, as shown in a previous paper (Owen and Vaughan, 1959) the long range of the β-particle from $^{90}Sr + {}^{90}Y$ results in a considerable radiation dose being received throughout

all the bone and marrow and surrounding tissue following even a single injection. These factors make the interpretation of the results difficult and it is not possible from our experiments to give any precise answer to the interesting questions, what is the tissue, how large a volume must be irradiated and how great a dose must be received in order to produce a bone tumour.

There are strong indications from the damage described in these rabbits in the previous two papers, that it is the osteogenic connective tissues covering the bone surfaces (this includes the surfaces of the canals and cavities within the bone wall as well as the periosteal and endosteal surfaces) which are important from the point of view of tumour production. This tissue is, of course, widely distributed throughout the bone. There appeared, however, to be some correlation between the different patterns of damage and accumulated dose in the two cases studied. In the injected animals the maximum value of the accumulated dose and the greatest damage were limited to a small region of the bone, in the fed animals they were widespread over a large length of the bone examined.

Until more is known of the mechanism of the production of tumours it will be impossible to determine exactly the site of tumour origin, if indeed the latter term has any real meaning. Furthermore, it is also too simple an approach to a complex problem to try to relate damage to accumulated dose only, since it is well known that dose-rate is also important; this is confirmed in the present experiments as is discussed below.

It appeared that some of the most striking differences in damage could be attributed to the differences in dose-rate to the relevant tissues. The rabbits given a single injection received an initial heavy dose-rate to the tissues just below the epiphyseal plate at the time of injection, which then falls off with time. In the rabbits fed ^{90}Sr daily, the dose-rate builds up gradually from zero to a maximum with time. The damage produced in both cases is severe but different. In the case of the single injection there is gross interference with cartilage resorption together with abnormal bone formation in association with the limited region of maximum dose-rate. Much of this bone and cartilage is dead but on the periphery there is found abnormal bone which is living. In the case of the fed animals where the dose-rate is lower and more uniform throughout the bone there is no obvious failure of the normal process of resorption and no excess of dead bone. Abnormal bone formation is present but is more widespread, occurring along the length of the endosteal surfaces. Tumours, however, were the end result in both cases. It is well known that tissue damage is dependent on dose-rate as well as total dose and our experiments are in agreement with this.

SUMMARY

1. The dose-rate pattern in bone and marrow of the upper half of the tibia has been measured in rabbits given a single injection or started daily feeding of ^{90}Sr at the age of 5–8 weeks.

2. The patterns of accumulated dose and of dose-rate have been compared with the patterns of radiation damage found in the two cases.

3. In the animals given an injection of ^{90}Sr the maximum value of the accumulated dose and the greatest damage were limited to a small region of the bone at the level of the epiphyseal plate at the time of injection. In the animals fed ^{90}Sr the maximum value of the accumulated dose was received over a large length of

the bone examined and the greatest damage was found widespread along the endosteal surfaces. Both regions of greatest damage appeared to correspond to the sites of tumour origin.

4. Some features of the damage could be related to the different patterns of dose-rate. In the injected animals the dose-rate fell from an initial high value, in the fed animals the dose-rate increased gradually from a low value. In the case of the injected animals the initial high dose-rate caused gross failure of cartilage resorption and inhibition of longitudinal and transverse growth. No obvious failure of resorption or stunting of growth were seen in the fed animals.

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REFERENCES

BRUES, A. M.—(1949) *J. clin. Invest.*, **28**, 1286.
DOWNIE, E., MACPHERSON, S., RAMSDEN, E., SISSONS, H. AND VAUGHAN, J.—(1959) *Brit. J. Cancer*, **13**, 408.
FINKEL, M. P.—(1956) *Radiology*, **67**, 665.
Idem, LISCO, H. AND BRUES, A. M.—(1955) Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory, ANL-5378, 106.
OWEN, M., JOWSEY, J. AND VAUGHAN, J.—(1955) *J. Bone Jt. Surg.*, **37 B**, 324.
Idem, SISSONS, H. AND VAUGHAN, J.—(1957) *Brit. J. Cancer*, **11**, 229.
Idem, AND VAUGHAN, J.—(1959) *Brit. J. Radiology*, in press.
TUTT, M., KIDMAN, B., RAYNER, B. AND VAUGHAN, J.—(1952) *Brit. J. exp. Path.*, **33**, 207.

CARCINOGENESIS BY CHOLESTEROL

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THE existence of carcinogens of biological origin was demonstrated for the first time by Shabad (1937) who induced sarcoma in mice by injecting lipoid extracts from post-mortem human tissues. His discovery was confirmed by a number of other investigators (des Ligneris, 1940; Hieger, 1940; Steiner, 1941; Sannié, Truhaut, Guérin and Guérin, 1941) who found that sarcomas in mice resulted from the subcutaneous injection of cholesterol-rich extracts, e.g. (1) the unsaponifiable part of the fatty fraction of liver, kidney, muscle or lung of human subjects who had died of cancer or of some other cause, or (2) the unsaponifiable fraction from the brain and spinal cord of cattle. The succeeding steps in this work showed that cholesterol itself, even when highly purified had carcinogenic activity (Hieger, 1947, 1949, 1957; Hieger and Orr, 1954). In a recent publication, Hieger (1957) describes an experiment in which 11 mice developed sarcoma out of 115 (initially) injected mice; if the incidence is calculated on the number of mice which survived to the minimal latent period (thus cancelling out from the initial number all mice which died early and were not at risk) the incidence amounted to 14 per cent. The cholesterol in this experiment was highly purified (Schwenk process) and administered as a 10 per cent solution in olive oil prepared by heating on the water bath. In control experiments where the solvent alone was injected into mice, (lard was used as solvent in the earlier tests and controls), 5 sarcomas developed in a total of 1122 treated mice (Table I).

TABLE I.—*Control Tests on Solvents Injected Subcutaneously into Mice*

Solvent	Strain of mice	Number at start	Survivors (months) 12	18	24	Sarcomas
Lard	stock, C_{57}	366	159	81	16	1
,,	stock	100	47	20	2	0
Olive oil	C_{57}	134	90	45	6	1
,,	stock	50	33	12	2	0
,,	,,	50	24	13	0	0
,,	,,	66	14	1	0	0
,,	,,	56	47	20	1	1
,,	C_{57}	50	39	28	13	—
,,	C_{57}, stock	58	52	33	7	0
Tricaprylin	C_{57}	30	17	6	2	1
Stearic acid + olive oil	stock	51	32	13	2	0
Tristearin + olive oil (5 : 30)	,,	50	33	13	3	1
Sesame oil	,,	61	46	13	4	0
Total		1122				5

The reports of the carcinogenic activity of cholesterol have provoked comment and criticism. For example, (I) it has been objected that since cholesterol is present in all tissues of the body and particularly in atheromatous arteries, cancer should be expected to occur in all tissues and particularly at atheromatous sites. (II) A second criticism takes another form ; since cholesterol is found in all tissues but cancer does not appear in every tissue, the carcinogenic activity of cholesterol when injected into mice could be due to an active derivative formed from the cholesterol by some reactions peculiar to the conditions of the experiment. (III) A third objection suggests that as the threshold carcinogenic dose of benzpyrene for the mouse is of the order of only a microgram the activity of cholesterol in the mouse experiments could be due to contamination with a minute trace of a potent carcinogen of the type of benzpyrene. (IV) A fourth comment points out the difficulty of reconciling the negative results obtained by other workers who tested cholesterol by more or less the same technique which gave positive results in Hieger's experiments. (V) Fifthly, the idea has been put forward that since watery suspensions of cholesterol have proved negative when tested for carcinogenic activity and since in tests giving positive results the cholesterol is heated with oily solvent at water bath temperature for up to 2 hours before the solution is injected, chemical changes could well take place during the heating which might at least contribute to the carcinogenic activity of the solution.

These five questions will now be discussed.

(I) *Cholesterol present in all tissues of the body*

Cholesterol is found associated with fat in all tissues and particularly in atheromatous aortas, yet cancer in man develops at sites of which the aorta is one of the least common. The apparent contradiction between this fact and the positive mouse tests on cholesterol is by no means insoluble. Urethane when given to mice in the drinking water or painted on the skin, gives rise to tumours of the lung only ; β-naphthylamine when inhaled or absorbed through the skin by men working in the dye and rubber industries causes cancer of the bladder and not of other organs ; benzpyrene applied in large doses over a space of several years failed to induce cancer in the skin of the monkey. When discussing carcinogenic action, the tissue, and the species of animal to which it belongs as well as the type of carcinogen must all be taken into consideration. The human aorta contains very little cellular tissue which may well be resistant to carcinogenesis by cholesterol. The inferences which can be made from the work on cholesterol are not intended to suggest that all human cancer is mediated by cholesterol, no such crude hypothesis is propounded here, the illustrations just quoted merely show that the *opposite* conclusion has not been established.

(II) *Injected cholesterol as the precursor of a carcinogen*

In its simplest form, question (II) suggests that although cholesterol itself is a normal chemical component of the tissues, when it is injected subcutaneously in oily solution it is slowly converted to some active derivative which is the true carcinogen.

This possibility gains support from Bischoff's experiments on the reported carcinogenic capacity of oxidative derivatives of cholesterol which he obtained as a by-product in the preparation of progesterone by the oxidation of cholesterol

with permanganate. The mixture of compounds resulting from the reaction induced sarcomas on injection subcutaneously into mice of the Buffalo strain. From this result Fieser *et al.* (1955) concluded that the active agent in carcinogenesis by cholesterol was some ketonic derivative (e.g. Δ^4-cholestene-3-one) or a chemically active isomer, namely, lathosterol (Δ^7-cholestenol). Fieser *et al.* (1955), Bischoff and Rupp (1946), Bischoff, Lopez and Rupp (1954), and Bischoff *et al.* (1955) reported that a number of derivatives of cholesterol when injected in batches of 30 Buffalo mice induced 19–60 per cent of fibrosarcomas. The most potent member of this group of compounds was 6β-hydroperoxy-Δ^4-cholestene-3-one. They state that purified cholesterol was found inactive but that unpurified cholesterol had some carcinogenic potency.

> " Administered in sesame oil, impure cholesterol lathosterol, and the non-keto fraction of cholesterol oxidized by oxygen in soap suspension produced higher incidences of fibrosarcoma than controls which received only sesame oil or sesame oil plus pure cholesterol or cholestenone."

The results of Bischoff and his colleagues are summarised in Table II.

TABLE II.—*Oxidative Derivates of Cholesterol as Carcinogenic agents (Fieser, et al., 1955 ; Bischoff, et. al., 1955)*

Compound tested	Per cent fibrosarcomas (in groups of 33 Buffalo mice)
none, control	0
6-hydroxy-Δ^4-cholestene-3-one	19
Δ^4-cholestene-3,6-dione	34
none, control	0
cholesteryl oxide	43
6-hydroperoxy-Δ^4-cholestene-3-one	60

Some of the compounds reported to be strongly carcinogenic by Fieser and Bischoff have been tested in the writer's laboratory. The yield of tumours does not confirm the statement of the American workers that these oxidative derivatives are more potent carcinogens than cholesterol, neither can we agree with their pronouncement on the lack of potency of cholesterol. It must be noted however that different strains of mice were used in the two laboratories.

In an earlier paper (Hieger, 1959) giving the results of experiments in progress it was stated that none of the compounds tested had at that stage produced sarcomas ; the experiments are now almost completed and a number of tumours have developed, 5 of them in the series of mice injected with hydroperoxide.

The results are shown in Table III ; they indicate that (1) under our conditions only the hydroperoxide of all the oxidative derivatives of cholesterol has given evidence of any appreciable carcinogenic activity ; (2) mice of C_{57} strain are more sensitive than stock mice, but this difference probably depends to some extent on their longer life span. The same difference of response between C_{57} and stock mice was observed when two parallel series were tested with cholesterol purified by the Schwenk process (Table IV). Although C_{57} mice were used for the tests on Δ^5 : cholestene-3-one and lathosterol, the yield of tumours totalled a single sarcoma ; the crude $KMnO_4$ oxidation product, Δ^4-cholestene-3 : 6-dione and 6-hydroxy-Δ^4-cholestene-3-one were not tested on C_{57} mice and it is possible that they too might have given some positive results under such conditions.

TABLE III.—*Sarcoma Induction by Derivatives of Cholesterol*

Compound	Number of mice and strain	Survivors (months) 12	15	18	21	24	30	Sarcomas
Crude $KMnO_4$ oxidation product of cholesterol	stock 50	24	17	14	3	1	0	0
Δ^4-cholestene-3 : 6-dione (in olive oil)	,, 55	31	22	16	8	4	0	0
6β-hydroperoxy-Δ^4-cholestene-3-one (in olive oil)	,, 55	28	22	18	11	5	0	0
(In sesame oil)	,, 65	32	27	22	14	6	—	0
,,	,, 51	34	31	15	8	2	—	0
,,	Buffalo 36	7	4	2	1	0	—	1
,,	C_{57} 50	46	39	38	23	15	—	4
6-hydroxy-Δ^4-cholestene-3-one (in olive oil)	stock 50	35	26	13	9	0	—	1
(in sesame oil)	,, 54	30	23	21	4	0	—	1
Δ^5-cholestene-3-one (in olive oil)	,, 40	14	12	8	4	0	—	0
,,	C_{57} 50	38	34	29	23	11	—	1
Lathosterol	C_{57} 50	39	—	17	—	5	—	0

TABLE IV.—*Sarcoma Induction by Purified Cholesterol (Schwenk Process) Injection in C_{57} Mice Compared with Negative Results in Stock Mice.*

No. of mice	Strain	Survivors (months) 12	18	24	30	Sarcomas
57	Stock	25	14	5	1	0
50	C_{57}	47	31	24	7	3 (latent periods 12, 18, 24 months)

As far as the results go they suggest that the hydroperoxide and pure cholesterol have about the same order of carcinogenic activity (Tables III and IV) which gives no support to the idea expressed by Fieser *et al.* (1955), and is implicit in the writings of Bischoff *et al.* (1955), that cholesterol is a pre-carcinogen when injected into the mouse and has to be converted to an oxidised carcinogenic derivative before it can induce sarcoma.

(III) *Trace contamination by carcinogens of hydrocarbon type*

The experiments designed to test lipoid tissue-extracts and the earlier tests with cholesterol before 1950 were carried out in a room at the Chester Beatty Institute where some other series of mice were being treated with potent carcinogens such as benzpyrene, thus introducing the possibility of contamination. In 1950 all the experiments on carcinogenesis by lipoids and steroids were transferred to our country laboratories, twenty miles out of London, where the atmosphere is less smoky and where there are arrangements for housing the mice in rooms uncontaminated by carcinogens other than cholesterol (Hieger and Orr, 1954). Nevertheless, it soon became clear that even if all reasonable precautions were taken to avoid contamination, cholesterol still induced sarcoma in mice. In order to obtain some data on the degree of contamination which might appreciably affect the yield of sarcomas with cholesterol alone, series of mice were given small doses of benzpyrene to find where the threshold dose of benzpyrene lay (Table V).

The results of the experiments detailed in Table V suggest that (1) the threshold dose of benzpyrene is of the order of 1 γ for C_{57} mice or stock mice, (2) an amount

of benzpyrene in the region of 10 γ would have to be injected along with the cholesterol in order to give a yield of sarcomas appreciably higher than what would be expected with cholesterol alone, (3) the sensitivity of mice to small doses of benzpyrene can vary by a factor of at least 5 to 1.

TABLE V.—*Threshold Dose of Benzpyrene Required for Sarcoma Induction*

Experiment	No. of mice at start	Strain	Total dose of benzpyrene (in 10% cholesterol : 90% olive oil)	How administered	No. of sarcomas
1	50	C_{57}	0·04 γ	in a single dose	0
2	,,	,,	0·4 γ	,,	1
3	,,	,,	4·0 γ	,,	5
4	,,	,,	40·0 γ	,,	23
5	,,	stock	7·5 γ	divided in 6 injections	2
6	,,	,,	0·75 γ	,,	3
7	,,	,,	0·075 γ	,,	3
8	,,	,,	50 γ	,,	19
9	,,	,,	5 γ	,,	3
10	,,	,,	0·5 γ	,,	0
11	,,	,,	0	,,	0
12	,,	,,	0	,,	0
13	,,	,,	10 γ	,,	1
14	,,	C_{57}	10 γ	,,	10
15	,,	stock	10 γ	in single dose	6

If it be assumed as a working hypothesis that sensitivity is proportional to the yield of tumours and inversely as the dose of carcinogen required, experiments 3, 13, 14 and 15 (Table V) suggest that C_{57} mice are of the order of 10 times as sensitive as stock mice to threshold doses of benzpyrene when it is administered in 5 divided doses, but only about twice as sensitive when the dose is given in one injection ; experiments 9 and 13 suggest that the sensitivity of different lots of stock mice can vary by something of the order of 6 : 1 or more. Although the benzpyrene dose varied in the ratio 1 : 100 in experiments 5, 6, 7, the yield of tumours was much the same, suggesting that the cholesterol in the injection material was the active carcinogen and not the benzpyrene. In these three experiments the incidence was about 6 per cent calculated on the number of mice at start, but about double this figure when the calculation is based on the mice which survived a minimum of 1 year and therefore were effective, (i.e. at risk). An incidence of 10–15 per cent would agree well with that obtaining in several of our experiments where the mice developed a reasonable proportion of tumours when injected with cholesterol (the frequently occurring incidence of about 10–15 per cent will be further discussed in a forthcoming publication). This figure of 10–15 per cent can now be contrasted with the results for experiments 10, 11, 12 where the incidence was 0 per cent. As far as the data go, they suggest that the sensitivity of different batches of mice can vary over a considerable range whether this sensitivity is assayed by the use of cholesterol or by small doses of benzpyrene.

To extend the idea of possible contamination by benzpyrene a further step, the following experiment was carried out : mice were injected with cholesterol and arranged in groups of 4 in separate cages ; to each cage was then added a single mouse of a different colour (a coloured mouse with 4 white mice or a white mouse with 4 coloured mice) which was painted biweekly with benzpyrene solution in the interscapular area during the whole course of the experiment. The

mice injected with cholesterol were thus in an environment contaminated with benzpyrene, yet only in experiment A (Table VI) was there a large number of sarcomas which would be attributed to benzpyrene. In this particular experiment, the 11 injections of cholesterol were given at 4 weekly intervals concurrently with the biweekly painting of the single mouse with benzyprene. A possible explanation of the results in experiment A is that at the injection of cholesterol some benzpyrene on the fur, transferred there by contact with the painted mouse, was pushed through the skin into the subcutaneous layers. In a few cases papillomas arose on the skin of the mice which had not been painted with benzpyrene but had a cage-mate which was so treated.

TABLE VI.—*Effect of Exposing Mice Injected with Cholesterol to an Environment Contaminated with Benzpyrene*

In each experiment below the mice were caged in groups of 5. Four mice were for injection *only*, and the fifth for painting *only* (with benzpyrene).

			Survivors			
				12	18	24
		Strain	Start	(months)			Sarcomas
A	Injected 11 times with 10 per cent cholesterol in olive oil at beginning of each of 11 months. In the 2nd and 3rd week of each month—biweekly painting	stock	80	42	16	—	12
B	4 injections (cholesterol) at fortnightly intervals, then after a 3-week interval painting begun	C_{57}	40	30	26	3	0
C	4 injections (cholesterol) at fortnightly intervals, then after a 3-week interval—painting begun.	stock	40	30	13	2	2
D	4 injections (cholesterol) at fortnightly intervals, then after 4 months—painting begun.	stock	40	28	12	2	0
E	Paint biweekly for 3 months only. Then after an interval of 1 month, 4 fortnightly injections of olive oil—tristearin (5 : 1).	stock	40	12	2	—	0
F	Inject 4 times with an olive oil : tristearin mixture (5 : 1), then after an interval of 3 weeks, biweekly painting begun.	stock	44	28	14	2	2
G	Painted biweekly for 3 months. Then, after an interval of 2 weeks given 4 injections of cholesterol.	stock	60	11	—	—	0

EXPLANATION OF PLATES

FIG. 1.—Sarcoma induced at site of injection of purified cholesterol in olive oil. ♀ stock mouse. 16th month. × 65.

FIG. 2.—Same tumour as in Fig. 1. × 300.

FIG. 3.—Sarcoma induced at site of injection of purified cholesterol in olive oil. ♂ stock mouse. 18th month. × 65.

FIG. 4.—Same tumour as Fig. 3. × 300.

FIG. 5.—Sarcoma induced at site of injection of purified cholesterol in olive oil. ♂ stock mouse. 9th month. × 75.

FIG. 6.—Same tumour as in Fig. 5. × 300.

FIG. 7.—Tumour on *left* side of ♀ stock mouse which had been injected on *right* side with tristearin in olive oil. 19th month. × 90.

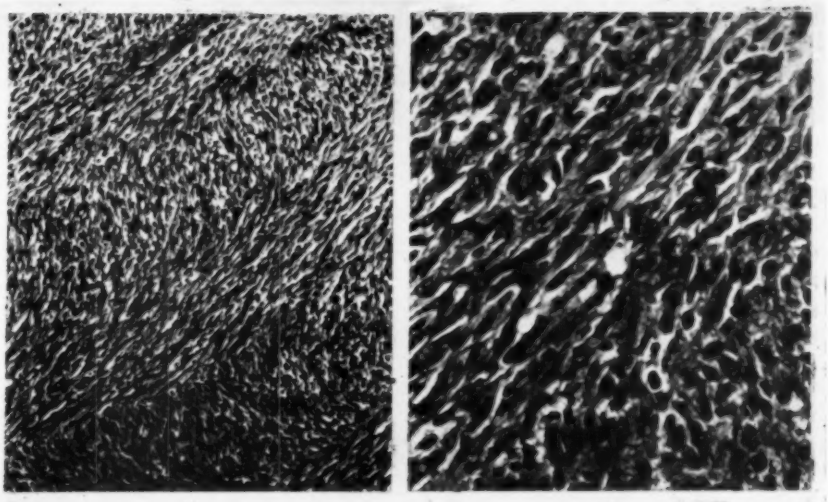
FIG. 8.—Same tumour as in Fig. 6. × 300.

FIG. 9.—Tumour on *left* side of ♀ stock mouse which had been injected on *right* side with cholesterol in olive oil containing added 1 per cent turpentine. 19th month. × 90.

FIG. 10.—Same tumour as in Fig. 9. × 300.

FIG. 11.—Tumour on *left* side of ♂ stock mouse which had been injected on *right* side with cholesterol in olive oil. 15th month. × 90.

FIG. 12.—Same tumour as in Fig. 11. × 300.



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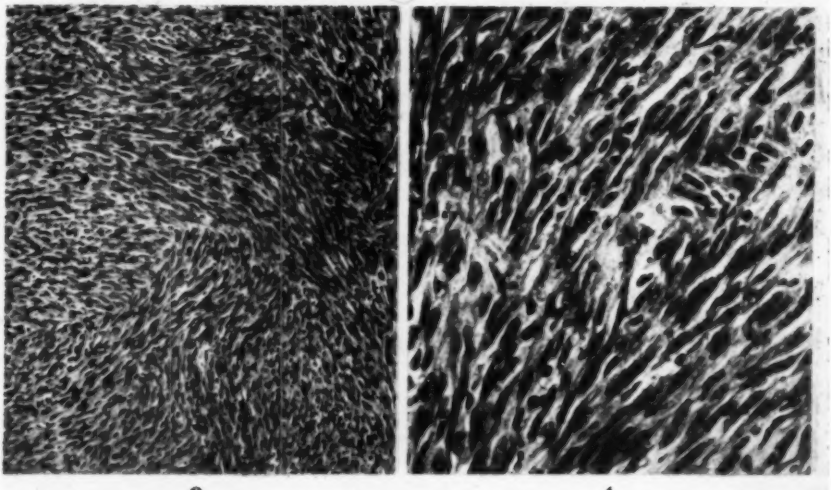

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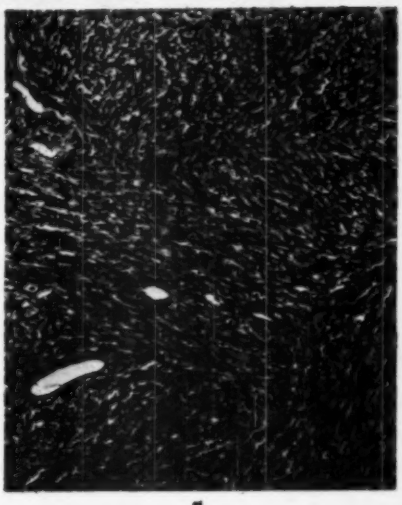

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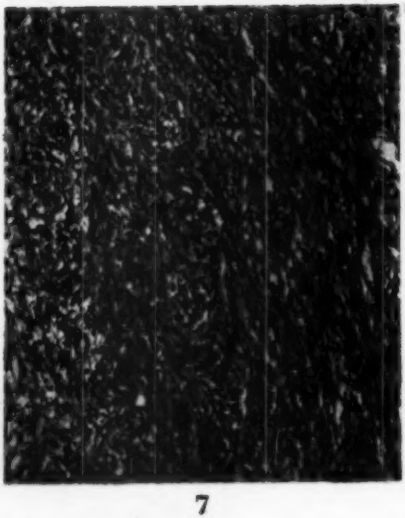

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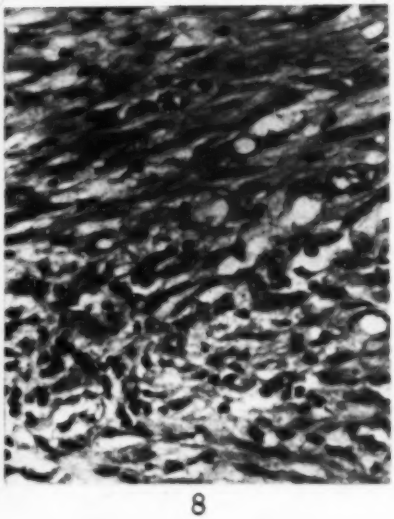

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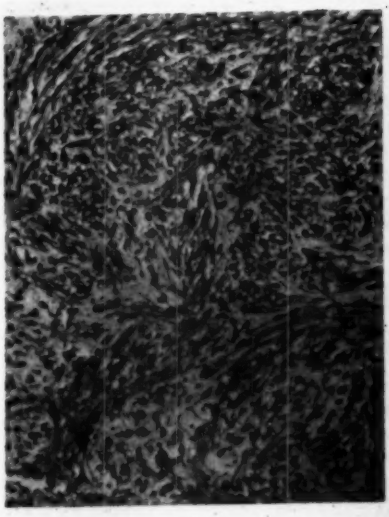

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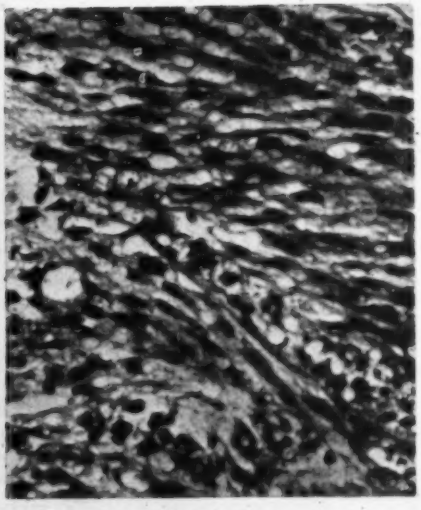

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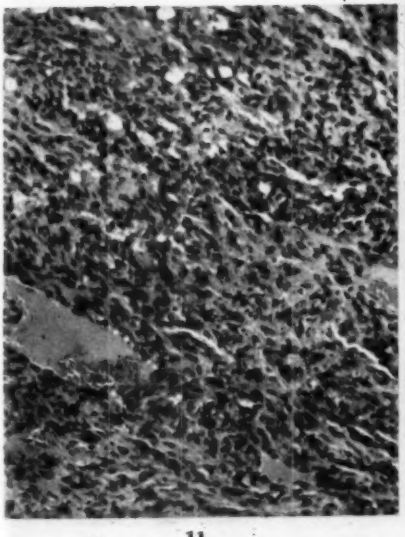

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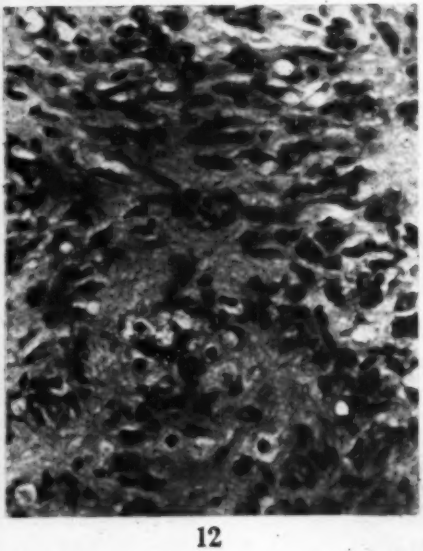

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These data show that no very stringent precautions need to be taken to avoid fictitious results due to contamination, since the mice in experiments B and C, although in an environment contaminated with benzpyrene, did not develop sarcomas in excess of what would be expected from the injection of cholesterol *per se*. It is difficult to explain the appearance of the two sarcomas in experiment F (Table VI) where no cholesterol but a mixture of two fats were used for injection (olive oil and tristearin) in order to test the possible localising effect of lipoid substances other than cholesterol.

(IV) *In other laboratories, cholesterol has not produced tumours*

Hartwell (1951) quotes 39 entries under the section dealing with tests for the carcinogenicity of cholesterol; some details are given of the species of experimental animal, vehicle and dose of cholesterol, site and route of administration, duration of experiment, survival rate and yield of tumours. None of these 39 experiments gave positive results of the kind which are the criteria of the present enquiry, i.e. spindle-celled sarcoma at the site of injection (sub-cutaneous) in mice injected with large doses of cholesterol of the order of a minimum of 40 mg. administered in lard or olive oil, in mice of stock or C_{57} strain which had a satisfactory survival rate. However, Hartwell's compilation was not completely up to date even in 1951 since he does not refer to the writer's experiments published in 1947 where 11 sarcomas were obtained by the injection of commercial cholesterol in 144 mice (Hieger, 1947).

As far as the author is aware, his experiments are almost the only ones giving positive results in tests for the carcinogenic activity of cholesterol. Is the discrepancy due to differences in the solvent, in the strain of mouse, in the duration of the experiment or in the environmental conditions of the animals? The role played by the solvent in carcinogenesis by lipoid substances and steriods has not yet been settled. Cholesterol of different degrees of purity has been found highly active when administered in olive oil or in lard; Steiner (1941) reported that mixtures of the unsaponifiable fractions of human liver with tricaprylin or with sesame oil were strongly carcinogenic; Bischoff *et al.* (1955) tested oxidative derivatives of cholesterol suspended in sesame oil with positive results. These four oils (lard, olive oil, sesame oil and tricaprylin) can accordingly be considered as suitable vehicles for testing carcinogens of lipoid or steroid type since the control tests on the oils alone give a very low yield of tumours, namely, of the order of $\frac{1}{2}$ per cent (Table I).

The choice of the strain of experimental mouse is probably an important factor in affecting the response to lipoid carcinogens. Cholesterol in olive oil or in lard has given an incidence of 10–15 per cent or more of sarcomas in C_{57} mice, in mixed commercial stock mice, and in the laboratory stock bred from a mixed commercial stock. In an experiment on 100 mice of the CBA strain injected with commercial cholesterol in olive oil, the yield of sarcomas was less, namely 2 per cent (Table VII). Small series of C_3H mice tested with the same solution proved refractory and small series of mice of the MRC strain reacted positively.

Such differences of sensitivity of different strains of mice to cholesterol as a carcinogen are not unexpected since variations of susceptibility to conventional carcinogens (e.g. hydrocarbon type) are well known and an example is shown in Table V. What is more difficult to explain is the frequently occurring fluctuations in response exhibited by different batches of mice of the same genetic origin.

Differences of sensitivity to carcinogens can easily be found when a minimal dosage of carcinogen is used but when an excessive dose of carcinogen is applied (such as, say, a milligram or even a tenth of a milligram of benzpyrene) anything but gross differences of sensitivity will be masked by the excess of carcinogen; therefore, in order to reveal and assess the comparative sensitivity of different groups of mice, the dose of carcinogen should be at threshold, or near threshold, level. If the carcinogen is of an order of potency much below that of the hydrocarbon carcinogens of the type of benzpyrene, it can be expected that even after the application of large doses, only a fraction of the mice would react and the conditions would be well suited for demonstrating variations of susceptibility which are not likely to appear when hydrocarbon carcinogens are employed, unless in minute doses. In Table VII are shown the fluctuations of response when mice of C_{57} or stock strains were tested with pure or with commercial cholesterol. The yield of sarcomas varied from 0–14 per cent or more in different batches of mice. Experiments t and q (both groups injected with commercial cholesterol) are of special interest: the origin of the mice was the same (a more complete correspondence would have been achieved if the individual members of the two groups had been thoroughly "shuffled"). Group q was housed in a room kept at about 70° F. and water was supplied in bulbs in the usual way: group t was housed in cages well supplied with wood wool, in a room facing north where the temperature followed the external temperature but was not allowed to go below 40° F. and the only water available to the mice was supplied in cabbage leaf. In experiment q the yield of sarcomas was zero in 50 mice, in t it was 5 in 46 mice (initially) amounting to an incidence of 16 per cent calculated on the survivors at one year.

These experiments suggest that environmental factors were of importance in influencing the response to the carcinogen. A comparison of experiments s (pure cholesterol) and q (commercial cholesterol) suggests that purified cholesterol (Schwenk process, see Hieger (1957) for details) is more potent carcinogenically than commercial cholesterol; but it must be noted that in experiment r no sarcomas were induced in 54 stock mice with the olive oil preparation of pure cholesterol, whereas in n and o the incidences were 14 per cent in one case and was calculated to have been the same theoretically in the other (Hieger, 1957). It is very difficult to find any explanation of why the same, or very nearly the same, preparation of cholesterol gives 14 per cent of sarcomas in stock mice in one experiment and 0 per cent in another.

The conclusions to be drawn from the data shown in Table VII are that no single variable—nor the purity of the cholesterol, nor chance contamination with atmospheric carcinogens nor strain differences, nor solvent differences—can be implicated as solely responsible for the fluctuations in carcinogen response; the alternative explanation is that a combination of some genetic, congenital or environmental factors (e.g. temperature and drinking water) are the effective agents, but of course such an explanation has still to be proved adequate.

In Section III the range of variations in susceptibility to threshold doses of benzpyrene was observed to be something of the order of 5 : 1, if the sensitiveness be regarded provisionally as proportional to the percentage yield of tumours and inversely as the dose of carcinogen. Fluctuations of susceptibility of this order of magnitude could explain why some batches of mice do not react under the stimulus of cholesterol as carcinogen; as a first approximation it is suggested that such variations are due to the operation of the same factors which cause variations in

the incidence of "spontaneous" neoplasia in mice, although of course, there is no experimental evidence as yet for this idea. It will be recalled that Tannenbaum and Silverstone (1957) showed that mere alterations in the calorific value of the diet of mice could greatly influence the incidence of spontaneous mammary cancer and of tumours induced with carcinogens.

TABLE VII.—*Table Showing Incidence of Sarcomas in Mice After Subcutaneous Injection with Cholesterol*

Experiment	Institute	Room	Strain	Pure or commercial (comm.) Cholesterol	No. of mice and survivors (months)				Sarcomas	% Incidence on initial Nc.	Calculated on survivors at 1 year %
					Initial	12	18	24			
a	C.B.	—	C_{57}	comm.	108	68	45	10	10	9·3	14
b	,,	—	stock	,,	45	18	5	3	5	11	28
c	,,	—	MRC	,,	20	16	8	2	2	10	12
d	,,	—	C_{57}+ stock	pure	34	18	11	2	1	3	6
e	P.W.	non-C	C_{57}	comm.	60	28	14	3	1	1·7	3·6
f	,,	,,	stock	,,	70	38	18	2	3	4·3	7·9
g	,,	,,	,,	,,	65	57	41	11	3	4·6	5·4
h	,,	C	,,	,,	100	75	45	10	8	8	10·7
i	,,	,,	CBA	,,	100	71	45	2	2	2	2·8
j	,,	non-C	C_{57}	pure	172	126	81	20	5	2·9	4
k	,,	,,	,,	comm.	30	10	7	5	1	3·3	10
l	,,	,,	,,	,,	39	24	16	11	3	7·7	13
m	,,	C	,,	,,	75	46	26	3	4	5·5	8·7
n	,,	,,	stock	pure	115	66	22	10	11	9·5	14
o	,,	non-C	,,	,,	109	all † < 1 yr. (epidemic)			3	3	probably much > 3
p	,,	,,	,,	comm.	53	27	11	0	0	0	—
q	,,	,,	C_{57}	,,	50	38	32	23	0	0	—
r	,,	,,	stock	pure	53	25	12	2	0	0	—
s	,,	,,	C_{57}	,,	50	47	35	22	3	6	6·4
t	,,	low temperature	C_{57}	comm.	46	32	23	12	5	11	16
u	,,	,,	stock	,,	40	19	5	0	0	—	—

C.B. = Chester Beatty Research Institute, London. P.W. = Pollards Wood in Buckinghamshire, 20 miles out of London. Non-C = Non-Carcinogen Room. Carcinogens of conventional type (e.g. benzpyrene) were neither used nor kept in this room. C = Carcinogen room. In this room, besides mice injected with cholesterol, were other series undergoing treatment with benzpyrene. Low-temperature room = This room faces north; temperature not below 40°F. but otherwise follows outside temperature. Water is supplied only as present in fresh cabbage leaf.

Dunn, Heston and Deringer (1956) reported that in the female mice in their colony consisting of C_3H strain bearing the mammary milk agent, C_3Hf lacking the agent, $C_{57}BL$ strain and F_1 and backcross hybrids of these strains, a total yield of 106 subcutaneous fibrosarcomas were found in 4049 mice (initially). These workers quote Cloudman (1941) who, referring to a certain colony of mice stated that "sarcomas were among the more common malignant tumours in the subcutaneous regions, but there were no high-tumour stock, and it was unusual to find an incidence of over 15 per cent." Dunn, Heston and Deringer's (1956) results (106 tumours in 4049 female mice) represent a crude incidence of 2½ per cent sarcomas, but as the average latent period when the tumours occurred was at the advanced age of 22–32 months, which was approximately the average longevity of the mice, the true incidence was very probably a multiple of 2½ per cent,

say 5 per cent or more, since a fair proportion were dead before reaching the latent period and could not be considered at risk.

Some impression of the variations of incidence in their experiments is conveyed by the number of sarcomas in consecutive groups of C_3Hf mice (Table VIII).

TABLE VIII.—*Spontaneous Fibrosarcoma in C_3Hf Mice (Dunn, Heston and Deringer, 1956)*

Genetic composition of foster mother	Number of mice	Number of sarcomas
(a) C_3Hf mice foster-nursed on ⟶ C_{57} (100 per cent C_{57} chromatin : 0 per cent C_3H chromatin)	81	3
(b) C_3Hf mice foster-nursed on ⟶ Cross-bred (50 per cent C_{57} chromatin : 50 per cent C_3H chromatin)	90	9
(c) C_3Hf mice foster-nursed on ⟶ Cross-bred (25 per cent C_{57} chromatin : 75 per cent C_3H chromatin)	64	0

It is very difficult to explain how the variations in the genetic composition of the foster-mothers of the mice in experiments b and c was sufficient to change the incidence of spontaneous sarcoma from a maximum of 10 to 0 per cent.

The foregoing considerations suggest that (1) at low intensity stimulus (cholesterol as carcinogen, or threshold doses of benzpyrene or "spontaneous" sarcoma) several factors together or separately can cause wide fluctuations of incidence of sarcoma; (2) that of these factors, two can be identified namely the "environment" and the genetic composition of the mice; (3) that alternatively some other factors are operative, the nature of which is as yet unknown.

These 3 possibilities could explain the disagreement between the results of tests on cholesterol and its oxidative derivatives which were used by Bischoff, *et. al.* (1955) and by the writer. Bischoff *et. al.* (1955) and Fieser *et al.* (1955) report that 6β-hydroperoxy-Δ^4-cholestene-3-one is a potent carcinogen, for it gives sarcoma in over 60 per cent of Buffalo mice (Table II). In the tests described in Table III this compound is shown to be as active as cholesterol, i.e. giving a sarcoma incidence of 8–10 per cent but these experiments were carried out on C_{57} mice. A batch of Buffalo mice imported from the U.S. appeared healthy on arrival but have not maintained this condition, they are very prone to mite infestation (however, vigorous treatment with DDT and with benzyl-benzoate seems to be effecting some improvement of the state of the skin). Bischoff and Rupp (1946) find that this strain of mice is liable to spontaneous sarcoma, they state "... In previous experiments male mice observed for 20 months or more developed a higher incidence of skin tumours; namely 10 per cent in 30 control mice, and 23 per cent in 30 males that had received oestrone." Perhaps the Buffalo mice are specially sensitive to some carcinogens since their subcutaneous tissues are prepared, as it were, for neoplasia, assuming that the two processes follow the same path, but of course, their insensitivity to cholesterol as carcinogen would remain unexplained.

The spontaneous subcutaneous sarcoma rate in the mice in the author's laboratory must be very small indeed. An estimate of the incidence could be made by a count of the sarcomas which have appeared on the *left* side of the animals. All the injections of cholesterol, derivatives of cholesterol, lipoid fractions of tissues and controls on solvents, were carried out on the *right* side. To date over 140 sarcomas have been recorded on the right side and 7 subcutaneous tumours

on the left side, but the histological appearance of most of these 7 tumours is rather different from that of the spindle-celled sarcomas induced at the site of injection by cholesterol and by the lipoid tissue-extracts which are very similar histologically to the sarcomas produced by hydrocarbon-type carcinogens. Consequently, it is doubtful if there is any appreciable incidence of spontaneous spindle-celled sarcoma in our stocks. Photomicrographs of these two kinds of subcutaneous tumour are shown in Fig. 1–12.

Guérin (1954) states that in his colony of 6000 mice the most frequently occurring spontaneous tumour was not mammary cancer but connective tissue tumours of the reticuloendothelial system (275 cases equivalent to an incidence of 4 per cent). Thirteen spontaneous sarcomas were found, 12 of which were " fusohistiocytaire " ; the average tumour age was about 20 months.

V. *Role of heating as a possible contributory factor in carcinogenic activity of solution of cholesterol.*

Cholesterol as large dense flakes dissolves slowly on heating with olive oil at water bath temperature and when a large series of mice (say 50–100) are to be injected in turn, the time required for total heating may be approximately 2 hours. During that time chemical changes may occur in the solution ; indeed the solutions and even oils alone (particularly sesame oil and lard) after being heated on the water bath show a much increased spectroscopic absorption in the ultraviolet region of the spectrum.

Since our experiments with cholesterol administered in aqueous medium (i.e. finely ground cholesterol in suspension in 4 per cent gelatin gel) have so far given negative results, the criticism has been put forward that chemical changes taking place or initiated during the heating of the cholesterol with oil could explain the carcinogenic activity of the solution and the inactivity of the non-oily suspension. However, there is a total lack of experimental evidence for (and some facts can be advanced which do not support) this possibility. The control tests on the oily solvents alone (olive oil and lard) comprised over 1,000 mice which produced 5 sarcomas only. The oils were heated on the water bath before injection but since they were either liquid or melted very quickly, the time of heating was mainly dependent on the number of mice being injected and therefore the total heating could have been less than in the experiments with solutions of cholesterol ; but since the total number of injections was greater in the control series because oil alone is more readily dispersed in the tissues, the effective total heating was probably not very different in the two cases.

To pursue the same line of argument further, it would now be necessary to postulate that the presence of cholesterol during the heating is necessary for inducing the hypothetical chemical change. Sarcomas were not obtained in experiments set up to test this possibility (Table IX). Positive results must take precedence over negative results ; the latter may be due to an insensitive batch of mice or to environmental conditions which are not conducive to sarcoma production : some solutions of cholesterol heated in the usual way have *not* produced sarcoma in mice, and if for the sake of argument it be assumed that the agent is indeed formed during the heating, its hypothetical presence therefore is not as decisive as e.g. the sensitivity of the mice or the environmental factor. There seems no reason for multiplying the number of unknowns, and the formation of

a carcinogen by heating oil and cholesterol at water bath temperature remains at present unproved, what evidence exists is against such possibility.

TABLE IX.—*Results of Injecting* (1) *Heated Olive Oil*; (2) *Heated Solution of Cholesterol* (10 *per cent*) *in Olive Oil*

Number of Preparation	No. of mice	Strain	Survivors (months) 12	18	24	Sarcomas
(1)	12, 38	C_{57} stock 50	35	17	6	0
(2)	17, 33	C_{57} stock 50	35	15	4	0
(3)	14, 36	C_{57} stock 50	38	16	9	1

(1) Olive oil heated, with access of air, 75 hours on boiling water bath.
(2) 10 per cent cholesterol + 90 per cent olive oil heated with access of air, 75 hours on boiling water bath.
(3) Olive oil heated, with access of air, 76 hours on boiling water bath then 10 per cent cholesterol added without prolonged further heating.

SUMMARY AND CONCLUSIONS

1. Over 140 sarcomas in mice have been induced by the subcutaneous injection of oily solutions of (1) the unsaponifiable fraction of human tissue, or (2) of cholesterol.

2. Purified cholesterol is at least as potent as the commercial product before purification.

3. In one experiment on highly purified cholesterol, 11 sarcomas developed in 115 mice of which 66 lived over 1 year. If the mice which died early and were not at risk be omitted the incidence amounts to 14 per cent.

4. One of the oxidative derivatives of cholesterol reported by Fieser and Bischoff to be carcinogenic has given positive results when tested in the writer's laboratory. The most active of their compounds, 6β-hydroperoxy-Δ^4-cholestene-3-one, which is reported by Bischoff to give 60 per cent sarcomas in Buffalo mice, has induced sarcoma in 4 out of 50 treated C_{57} mice.

5. The degree of response to cholesterol as carcinogen has been found to fluctuate widely. It is suggested that such variations of sensitiveness are revealed by carcinogenic stimuli of low-intensity, and are linked with congenital and environmental factors.

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REFERENCES

BISCHOFF, F., LOPEZ, G. AND RUPP, J. J.—(1954) *Abstr. Amer. chem. Soc.* March 3–C.
Idem, LOPEZ, G., RUPP, J. J. AND GRAY, C. L.—(1955) *Fed. Proc.*, **14**, 183.
Idem AND RUPP, J. J.—(1946) *Cancer Res.*, **6**, 403.

FIESER, L. F., GREENE, T. W., BISCHOFF, F., LOPEZ, G. AND RUPP, J. J.—(1955) *J. Amer. chem. Soc.*, **77**, 3928.
GLOUDMAN, A. M.—(1941) Spontaneous neoplasms in mice. *In* ' Biology of the Laboratory Mouse ' (Snell, G. D. ed.). Philadelphia (Blakiston).
DUNN, T. B., HESTON, W. E. AND DERINGER, M. K.—(1956) *J. nat. Cancer Inst.*, **17**, 639.
GUÉRIN, M.—(1954) ' Tumours Spontanées des Animaux de Laboratoire '. Paris (Legrand).
HARTWELL, J. L.—(1951) ' Survey of compounds which have been tested for carcinogenic activity '. Bethesda, Md. (National Cancer Institute).
HIEGER, I.—(1940) *Amer. J. Cancer*, **39**, 496.—(1947) *Nature*, **160**, 270.—(1949) *Brit. J. Cancer*, **3**, 123.—(1957) *Proc. Roy. Soc. B*, **147**, 84.—(1959) *Acta Un. int. Cancr.* (in press).
Idem AND ORR, S. F. D.—(1954) *Brit. J. Cancer*, **8**, 274.
DES LIGNERIS, M. J. A.—(1940) *Amer. J. Cancer*, **39**, 489.
SANNNIÉ, C., TRUHAUT, R., GUÉRIN, M. AND GUÉRIN, P.—(1941) *Bull. Ass. franç. Cancer*, **29**, 106.
SHABAD, L.—(1937) *C.R. Soc. Biol., Paris*, **124**, 213.
STEINER, P. E.—(1941) *Cancer Res.*, **1**, 750.
TANNENBAUM, A. AND SILVERSTONE, H.—(1957) ' Cancer ' I (ed. R. W. Raven) London (Butterworths) p. 306.

THE *IN VITRO* AND *IN VIVO* ANALYSIS OF MAMALIAN TUMOUR VIRUSES

Experiments on the Epidemiology of the Polyoma Virus

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One of the basic problems in the viral origin of tumours is the mode of virus transmission. Such transmission may take place from one generation to another to give the appearance of a hereditary transmission, and between animals belonging to the same generation. Either of these factors may determine the spread of a tumour virus in a population, and the relative importance of one or the other may, of course, vary with different viruses. Information on the spread of virus correlated with the occurrence of tumours in the infected animals, can furthermore show to what extent animals may be infected with virus, fail to develop tumours, and yet transmit the virus in a way that will produce tumours in their progeny. Such studies can also show whether natural selection in an animal population can modify the virus's tumour inducing properties.

The polyoma virus (Stewart, Eddy and Borgese, 1958) is particularly favourable for such epidemiological studies. We have previously shown (Sachs *et al.*, 1959) that this virus can be transmitted between litter mates, from an infected mother to her litter, and from an infected litter to their mother. This virus can therefore be transmitted both from one generation to the next and between animals belonging to the same generation. In addition, since the presence of virus can be determined by the development of haemagglutination inhibition antibodies after virus infection of either newborn, young or adult animals (Sachs *et al.*, 1959 ; Fogel and Sachs, 1959), a rapid *in vitro* test is available for epidemiological investigations.

The present studies are concerned with some further experiments on the epidemiology of the polyoma virus, with particular reference to transmission between animals belonging to the same generation ; transmission during pregnancy and lactation, by sperm, and by tumour transplants ; and virus excretion in the urine, faeces and saliva.

EXPERIMENTAL

Transmission between animals

As in our previous study (Sachs *et al.*, 1959), the presence of virus transmission has been determined by tests for haemagglutination inhibition antibodies using guinea-pig red blood cells. The technique for the inhibition tests was the same as that used previously. 16 haemagglutinating units of virus were used and complete inhibition was taken as the end point. Serum from normal Swiss mice gave

inhibition titres up to 1 : 40, and very rarely 1 : 80. The Swiss mice used in these experiments were derived from animals that had been brother × sister mated for at least 20 generations. The virus stocks used were all tissue culture fluids obtained from infected mouse embryo monolayers.

It has previously been shown (Sachs *et al.*, 1959) that virus can be transmitted to mice kept together since birth (less than 24 hours old) with inoculated animals. Experiments were therefore undertaken on the possibility of transmission between adult animals. Swiss mice 6–7 weeks old were inoculated intraperitoneally with 1 ml. of polyoma virus with a haemagglutination titre of 128 per ml., and the inoculated animals then placed in the same cage as non-inoculated Swiss mice of the same age. One group of 5 inoculated plus 5 non-inoculated mice was kept in a room with no other virus inoculated animals, and another similar group was kept in a room which contained other animals inoculated with polyoma. The sera of all animals were tested at 7, 14, 35 and 63 days after inoculation and the data are given in Table I. These results show that although there was no evidence of virus transmission up to 35 days, transmission could be detected at 63 days between adult mice kept in the same cage. Non-inoculated adults kept in a different cage also showed at 63 days evidence of virus transmission if kept in the same room as other inoculated animals, but not if kept in another room (Table I).

TABLE I.—*Virus Transmission Between Adult Mice*

Mice tested	Room No.*	Haemagglutination inhibition titre 1 : at different intervals after contact with virus inoculated mice**			
		7 days	14 days	35 days	63 days
Virus inoculated mice	108	1280	640	2560	5120
	105	160	320	5120	5120
Cage mates of virus inoculated mice	108	20	20	20	160
	105	20	20	40	640
Room mates of virus inoculated mice	108	20	20	20	20
	105	40	20	40	320
Control non-inoculated mice		20	20	20	10

* Room No. 108 = room with no other virus inoculated animals.
Room No. 105 = room with other virus inoculated animals.

** Each inhibition test represents the pooled sera from 5 mice, and the same mice were tested throughout the experiment.

In order to further study the possibility of transmission between animals kept in different cages, tests were made on various mice kept as controls at different places in the same room (No. 105) as virus inoculated animals. The results (Table II) show that two out of eight groups tested had inhibition titres of 1 : 160. This therefore also shows that there had been some degree of virus transmission to the non-inoculated mice kept in different cages in the same room. All the cages and water bottles in this room have now been autoclaved and the room fumigated with formalin. Repeated tests made in two rooms next to the room with polyoma inoculated animals, and on animals kept on another floor, have so far shown no evidence of transmission to mice in these other rooms. The finding of tumours in non-inoculated mice kept in the same room as infected animals (Buffet *et al.*, 1958), suggests that virus has been transmitted between animals kept in different cages in the same room also in other laboratories (also Stewart, quoted in Buffet *et al.*, 1958, and Rowe *et al.*, 1958). The degree of transmission within a colony

will presumably depend on the methods used for cleaning the water bottles and cages, and handling the animals.

TABLE II.—*Tests for Contamination in Mice Kept in the Same Room as Virus Inoculated Animals*

Mouse strain	No. of mice tested	Treatment	No. of days in room with virus inoculated animals	Haemagglutination inhibition titre 1 :
Swiss	5 p	Inoculated as newborn with normal tissue culture medium	56	80
Swiss	5 p	,, ,, ,,	120	160
Swiss	5 p	,, ,, ,,	109	40
Swiss	6 p	,, ,, ,,	110	20
Swiss	5 p	Inoculated as newborn with normal adult Swiss kidney cells	51	80
Swiss	4 p	,, ,, ,,	50	160
Swiss	3 p	—	93	20
C57BL/6	2 p	—	35	10

p = pooled sera.

In view of the above findings, it is, of course, important to eliminate the possibility of chance contamination in experiments on virus transmission. It can be seen from the data on the time required for antibodies to develop in the cases of chance contamination, and from the titres obtained in our contamination results, that in none of the following experiments can the results be explained as due to chance contamination of virus from another source. As an additional precaution, control Swiss mice were kept next to the experimental animals, and tests for haemagglutination inhibition antibodies in these controls were invariably negative.

Virus excretion in urine, faeces and saliva.

Since it was found that virus can be transmitted between animals, experiments were carried out to determine the route of virus transmission. The first possibility examined was that of virus excretion in the urine. faeces and saliva. Cotton wool swabs were used to collect urine and to swab the mouth for saliva. The swabs were then placed in 2 ml. phosphate-buffered saline (Dulbecco and Vogt, 1954) containing penicillin and streptomycin for 3 hours at 4° C., the suspensions centrifuged in a clinical centrifuge at 2000 r.p.m. for 10 minutes, and the supernatant tested for virus. Faeces were also collected into 2 ml. of phosphate-buffered saline containing antibiotics, mixed, kept at 4° C. for 3 hours, the suspension centrifuged as for the urine and saliva samples and the supernatant tested. In order to test for virus in these supernatants, 0·75 ml. of each sample was inoculated intraperitoneally into two Swiss mice 5–7 weeks old, and the pooled sera from both mice tested for haemagglutination inhibition antibodies 14 days after inoculation.

Tests for virus excretion were made from two tumour bearing Swiss mice, one tumour bearing golden hamster, and four Swiss mice inoculated intraperitoneally as adults 70 days earlier with 1 ml. of virus with a haemagglutination titre of 128 per ml. In the tumour bearing animals as tested by palpation, one of the

mice (a female) had palpable presumably hair follicle tumours, the second mouse (a male) had a large presumably parotid tumour on one side, and the hamster (a male) had a large subcutaneous tumour. The adult inoculated mice were bled 7 days before they were tested for virus excretion and their sera gave haemagglutination inhibition titres of 1 : 5120.

The results obtained (Table III) show that virus can be excreted in the urine, faeces and saliva. In the two tumour bearing mice, one (the male) was at the time of testing excreting virus in the urine, faeces and saliva, whereas in the other virus was only detected in the urine. The tumour bearing hamster was negative for faeces and saliva. In the adult inoculated mice, virus excretion was detected in the urine and saliva in one mouse, in the urine only in two others, and in neither urine, faeces or saliva in the fourth mouse tested. In mice in which virus was excreted the urine was always positive.* The finding of excretion 70 days after virus inoculation into adults shows that the virus can multiply and that infected cells are releasing virus even in an adult inoculated animal. The presence of virus in the urine and mouth swabs of mice inoculated as newborn, has also recently been reported by Rowe *et al.* (1958).

TABLE III.—*Virus Excretion in Urine, Faeces and Saliva*

Animal tested	No. of animals	No. of days after virus inoculation	Haemagglutination inhibition titre 1 : in mice inoculated with*		
			Urine	Faeces	Saliva
Tumour bearing mouse	1	56	320	40	40
,, ,,	1	74	1280	160	320
Tumour bearing hamster	1	69	NT	40	40
Adult mouse inoculated intraperitoneally	1	70	320	40	320
,, ,,	1	70	40	20	20
,, ,,	1	70	1280	40	20
,, ,,	1	70	640	20	20

NT = not tested.
* Each inhibition test represents the pooled sera from 2 mice.

Transmission by sperm

The possibility of virus transmission by sperm was tested in tumour bearing Swiss mice, and in Swiss mice inoculated as adults intraperitoneally with 1 ml. of virus with a haemagglutination titre of 128 per ml., or intratesticularly (virus injected into one testis) with 0·05 ml. of virus with a haemagglutination titre of 1024 per ml. To collect sperm, the contents of both vas deferens were scraped with a spatula into 2 ml. of phosphate-buffered saline containing antibiotics. The debris was allowed to settle, and 1 ml. of the sperm containing supernatant of each sample was inoculated intraperitoneally into two Swiss mice 5–7 weeks old. The pooled sera of both mice were tested for haemagglutination inhibition antibodies 14 days later. As the material was collected from the vas deferens it includes in addition to sperm, secretions of the epididymis. The two tumour bearing mice

* In further tests on the urine of tumour bearing animals, virus excretion was detected in 10/10 mice with tumours, but in 0/10 hamsters with tumours.

tested had large palpable, presumably parotid tumours. All the mice from which sperm was collected had high inhibition titres (Table IV).

TABLE IV.—*Tests for Virus Transmission by Sperm**

Mouse tested	No. of mice	No. of days after virus inoculation	Haemagglutination inhibition titre 1 : Mouse tested	Haemagglutination inhibition titre 1 : Mice inoculated with sperm**
Tumour bearing	1	88	>40,960	40
,, ,,	1	99	>5,120	80
Adult inoculated intraperitoneally	1	73	5,120	40
,, ,,	1	73	5,120	40
Adult inoculated intratesticularly	1	42	5,120	40
,, ,,	1	42	>10,240	40

* Sperm was taken from the vas deferens so that this includes secretion from the epididymis.
** Each inhibition test represents the pooled sera from 2 mice.

The results (Table IV) have shown no evidence of virus transmission by sperm as measured by this test. Since a titre of 1 : 80 is on the borderline of significance for the presence of virus, the possibility of virus transmission by sperm is being examined by further experiments.

Transmission during pregnancy and lactation

The observation that virus can be transmitted from a mother to her litter (Sachs *et al.*, 1959) has been extended by experiments on the transmission of virus during pregnancy and lactation. Swiss mice were exposed to the virus for various periods during pregnancy and lactation, and the progeny tested for the presence of virus. The results are given in Table V.

TABLE V.—*Virus Transmission During Pregnancy and Lactation*

Litter No.	Number of mice tested	Virus exposure of litter (days) *In utero*	Virus exposure of litter (days) Suckling	Number of days after inoculatio of mother	Haemagglutination inhibition titre 1 : Mother	Haemagglutination inhibition titre 1 : Litter
1	4p	21	52	73	5120	640
2	3p	21	38	59	5120	2560
3	2p	6	34	40	>5120	80
3	1	6	34	53	NT	80
4	5p	5	22	27	1280	320
5	2p	3	38	41	2560	160
6	2p	3	38	41	>5120	40
6	4p	3	38	53	NT	80
7	2p	2	39	41	320	40
7	1	2	39	53	NT	80
8	4p	1	55	56	2560	20
9	2p	3	<1*	41	20	1280
9	2p	3	<1*	53	NT	2560
10	2p	3	<1*	41	10	10
10	1	3	<1*	53	NT	40

NT = not tested.
p = pooled sera.
* Foster nursed with non-inoculated mother within 12 hours after birth.

In the mice exposed for 21 days *in utero*, the mothers had received three intraperitoneal inoculations of 1 ml. of virus with a haemagglutination titre of 128 per ml. (at weekly intervals), and were then mated one week after the last inoculation. The time between the last inoculation and first day of pregnancy was 13 days for litter No. 1 and 27 days for litter No. 2. In both these litters (Table V) virus was transmitted to the progeny. In the other litters tested the mother was given one intraperitoneal inoculation of 1 ml. virus with a haemagglutination titre of 512 per ml. There has been virus transmission (Table V) in litters No. 4 (5 days *in utero*), 5 and 9 (3 days *in utero*); and no transmission in litters No. 8 (1 day *in utero*) and 10 (3 days *in utero*). Since a titre of 1 : 80 is on the borderline of significance in these experiments, there also does not seem to have been virus transmission in litters No. 3 (6 days *in utero*), 6 (3 days *in utero*) and 7 (2 days *in utero*). In the two litters tested after 3 days exposure *in utero* and then foster nursed on non-inoculated mothers, virus has been transmitted in litter No. 9 but not in litter No. 10.

The above results indicate that virus can be transmitted through the placenta. The effect of suckling is difficult to evaluate, since the litter may acquire the virus not only if it is present in the milk but from the material excreted in the urine, etc., by the mother. In order to overcome this difficulty, the milk is being directly tested both for the presence of virus and for antibodies against the virus produced by the mother.

TABLE VI.—*Haemagglutination Inhibition Antibodies in Mice Grafted with Tumour Cells*

Type of tumour	Host animals: Age at time of grafting	Host animals: Site of transplant	Number of mice tested	Number of days after grafting	Tumour growth at time of test*	Haemagglutination inhibition titre 1 : of grafted mice
Mammary adenoma	5	s.c.	2p	42	—	640
	7	,,	3p	42	—	1280
	9	,,	3p	42	—	320
Mammary adenoma	7	,,	1	50	+	640
	7	,,	2p	50	—	320
	28	i.m.	3p	45	—	640
Kidney sarcoma	28	,,	3p	45	—	1280
Kidney sarcoma	42	,,	3p	45	—	640
Parotid tumour	7	s.c.	3p	45	—	640
	42	i.m.	3p	45	—	160

s.c. = subcutaneous.
i.m. = intramuscular.
* + = growth of tumour transplant.
— = no growth of tumour transplant.
p = pooled sera.

Transmission by tumour transplants

Since it was shown that cells of polyoma induced tumours can continue to release virus (Sachs *et al.*, 1959), a possible source of laboratory contamination

with polyoma is its transmission by grafts of tumour cells. Mice grafted with tumour cells were therefore tested for haemagglutination inhibition antibodies. The results (Table VI) show that antibody titres were present, even in mice with no visible growth of the transplant. As the inoculated cells contained virus, this result is in agreement with expectation. These animals, like those inoculated with virus (Table III) may presumably excrete virus, so that the carrying of tumour transplants could be responsible for the spread of polyoma in the laboratory. It is of interest that with the mammary adenoma which gave both successful and unsuccessful transplants when inoculated into 7 day old hosts (Table VI), the animal with the progressive growth was a female, whereas those with no progressive growth were male. This therefore seems to have been a dependent tumour.

DISCUSSION

The results obtained in the present experiments have shown that virus can spread not only from newborn inoculated animals but also from animals inoculated as adults. This observation and the finding of virus excretion in adult inoculated mice 70 days after inoculation have also shown, that the virus multiplies and is emitted from infected cells in adult inoculated mice although so far no tumours have been observed in adult inoculated animals.. The polyoma is therefore a virus of presumably mammalian origin that can spread both from one generation to the next and between animals belonging to the same generation, and its mode of spread seems to resemble that found in avian lymphomatosis (Burmester, 1957). Vertical transmission (Gross, 1958) of the polyoma may therefore occur by infection of a litter by virus excreted in the urine, faeces or saliva, in addition to its transmission through the placenta and perhaps through the egg (although this last possibility has not yet been tested). The transmission of the polyoma seems to differ from that of the mammary tumour virus (milk agent) in which tests of urine and faeces have given no evidence of virus excretion (Dmochowski, 1953).

The finding of virus excretion in the urine and saliva would seem to be connected with the high frequency of kidney and salivary gland tumours produced by the virus. Cells of these two organs may thus be considered as among the main sites of virus multiplication in the animal. With the use of the plaque assay for the polyoma (Sachs, Fogel and Winocour, 1959 ; Winocour and Sachs, 1959) a single infective particle can be identified, so that quantitative studies on the *in vivo* sites of virus multiplication and the rates of virus excretion can now be undertaken.

The existence of virus transmission between animals even after birth makes it possible that animals may be infected with polyoma, fail to develop tumours, and yet infect their progeny at an early enough age for tumours to develop. The development of tumours may thus skip one or even several generations. Observations on virus transmission and tumour formation through several generations would also demonstrate whether the selection associated with natural transmission can change the tumour producing capabilities of the virus. Thus in the case of lymphocytic choriomengitis virus studied in a mouse stock for four years, there was during this period a marked decrease in the severity of the disease produced by the virus and a change in the mode of transmission (Traub, 1939). Such a reversion may perhaps also be found with the polyoma, and this could then

explain the absence of multiple tumours in the mice from which the virus was originally isolated.

Regarding the question of the origin of the tumour producing potentialities of the polyoma, it does not appear to be necessary to have long passage in tissue culture in order to obtain the multiplicity of tumours (Stewart *et al.*, 1957). Buffett *et al.* (1958) have also produced multiple tumours in mice by using the supernatant after high speed centrifugation of extracts from AKR mice with leukemia. It has furthermore been shown that extracts of organs from mice with polyoma induced tumours, and extracts of the tumours themselves, contain virus inhibitors (Fogel and Sachs, 1959), so that the removal of inhibitors, seems to be one of the requirements for multiple tumour production. Thus a variety of tumours have been produced in mice, hamsters and rabbits (Sachs *et al.*, unpublished) by virus, which seems to be free of inhibitors, released from polyoma induced mouse tumour cells in culture (Sachs *et al.*, 1959). There may, of course, be a change in virulence after serial passage similar to the type of change found with a pneumonia virus in mice which produced no symptoms as it occurred in [illegible]al mouse lungs, but produced fatal pneumonia after serial mouse lung passage ([illegible]sfall and Hahn, 1940). The polyoma may also be capable of returning to a provirus non-tumour forming state that requires activation. These possibilities can now be experimentally analysed with the use of *in vitro* methods. Epidemiological studies with this virus can thus demonstrate not only the mode of spread of the polyoma in a population, but they can also help to elucidate the basic question of the origin of its tumour inducing properties.

SUMMARY

A study has been made on some aspects of the epidemiology of the polyoma virus, using the formation of haemagglutination inhibition antibodies as a test for the presence of virus.

Experiments on transmission between animals have shown that virus can spread from adult inoculated mice to other adult mice kept in the same cage, and from inoculated to non-inoculated mice kept in different cages in the same room.

It has been shown that virus can be excreted in the urine, saliva and faeces. This observation, and the induction of a high frequency of kidney and salivary gland tumours by the polyoma, suggest that the cells of these two organs are among the main sites of virus multiplication *in vivo*.

Experiments on virus transmission during pregnancy have indicated that virus may be transmitted across the placenta.

Tests with sperm have so far given no evidence of virus transmission by this route.

Evidence has been presented to show that polyoma contamination in the laboratory may occur by the transplantation of polyoma induced tumour cells.

The finding of virus excretion in adult inoculated mice 70 days after virus inoculation, has shown that the polyoma can multiply in adult inoculated animals.

It is concluded that the polyoma can be transmitted both from one generation to the next and between animals belonging to the same generation. The existence of virus transmission from and to young and adult animals, makes it possible that animals may be infected with polyoma, fail to develop tumours, and yet transmit the virus in a way that will produce tumours in their progeny.

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REFERENCES

BUFFET, R. F., COMMERFORD, S. L., FURTH, J. AND HUNTER, M. J.—(1958) *Proc. Soc. exp. Biol. N.Y.*, **99**, 401.
BURMESTER, B. R.—(1957) *Ann. N.Y. Acad. Sci.*, **68**, Art. 2, 487.
DMOCHOWSKI, L.—(1953) *Advanc. Cancer Res.*, **1**, 103.
DULBECCO, R. AND VOGT, M.—(1954) *J. exp. Med.*, **99**, 167.
FOGEL, M. AND SACHS, L.—(1959) *Brit. J. Cancer*, **13**, 266.
GROSS, L.—(1958) *Cancer Res.*, **18**, 371.
HORSFALL, F. L., JR. AND HAHN, R. G.—(1940) *J. exp. Med.*, **71**, 391.
ROWE, W. P., HARTLEY, J. W., BRODSKY, I., HUEBNER, R. J. AND LAW, L. W.—(1958) *Nature, Lond.*, **182**, 1617.
SACHS, L., FOGEL, M. AND WINOCOUR, E.—(1959) *Ibid.*, **183**, 663.
Iidem, HELLER, E., MEDINA, D. AND KRIM, M.—(1959) *Brit. J. Cancer*, **13**, 251.
STEWART, S. E., EDDY, B. E. AND BORGESE, N. G.—(1958) *J. nat. Cancer Inst.*, **20**, 1223.
Idem, EDDY, B. E., GOCHENOUR, A. M., BORGESE, N. G. AND GRUBBS, G. E.—(1957) *Virology*, **3**, 380.
TRAUB, E.—(1939) *J. exp. Med.*, **69**, 801.
WINOCOUR, E. AND SACHS, L.—(1959) *Virology*, **8**, 397.

STUDIES OF POTENTIAL RADIOSENSITIZING AGENTS

An Effect of Tetrasodium 2-Methyl-1:4-Naphthohydroquinone Diphosphate (Synkavit) on the Ehrlich Mouse Ascites Tumour

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The use of tetrasodium 2-methyl-1:4-naphthohydroquinone diphosphate (Synkavit) as an adjunct to radiotherapy in the treatment of some inoperable malignancies in humans has developed from a laboratory study of this compound. Sensitization of cultures of chick fibroblasts towards X-irradiation as measured by mitotic inhibition and by production of chromosomal abnormalities (Mitchell and Simon-Reuss, 1947) has been confirmed (Mitchell and Simon-Reuss, 1952(*a*), 1952(*b*); Mitchell, 1955; Mitchell, Simon-Reuss and King, 1958). McKelvie (1959) has shown that the anti-mitotic action of X-rays on barley roots was increased by pretreatment with solutions of Synkavit.

However, since the radiosensitizing action on animal tumours has been disputed, a short review of the relevant reports follows. Mitchell's preliminary tests (Mitchell, 1952) indicated that repeated, large, intramuscular injections of Synkavit probably increased the number of permanent retrogressions of the primary tumour in rats carrying the Walker 256 carcinoma, but that the rather high number of spontaneous retrogressions (15 per cent) threw doubt upon the results. But the point was made, and this later proved to be vital, that fluorescence studies after intravenous injection showed that the maximum accumulation of the compound seemed to occur 30 minutes after injection. Fuller experiments followed (Mitchell, 1953). Here the spontaneous retrogressions had been almost eliminated by carrying the tumour as an ascites tumour before transplanting, and a single intravenous injection of Synkavit alone and in conjunction with 1100 r X-ray was studied. Two conclusions from these experiments are important. Firstly, the dose of X-ray alone was sufficient to produce 32/78 permanent retrogressions (40 per cent) and the radiosensitivity of this system was confirmed in another recorded experiment (120/274 permanent retrogressions—44 per cent). Secondly, the combination of 1100 r and intravenous Synkavit showed 52/77 permanent retrogressions (67·5 per cent), an obvious sensitization of this already radiosensitive tumour. Results of a quantitatively similar nature were given by Mitchell (1955) and fuller and more complete experiments on this aspect of our work are in the press (Mitchell, Simon-Reuss and King, 1959).

The most recent review of this problem is given by Tarnowski, Bane, Conrad, Nickson, Stock and Sugiura (1958). This includes an unsuccessful attempt to repeat Mitchell's experiments with the Walker 256 carcinoma. However, reference to their results (Table VIII, p. 245) shows that 1900 r gave an 8 week regression rate of only 9·8 per cent compared with untreated controls of 6·7 per

cent, and that 2300 r gave only a 20 per cent regression. It therefore follows that, in spite of a genuine attempt to repeat Mitchell's experiments, the New York group were in fact working with a much more radioresistant tumour. Failure to confirm Mitchell's work is therefore not surprising. Furthermore, the references quoted in this review showing findings at variance with Mitchell's are, in no case, comparable. Gellhorn and Gagliano (1950) were testing the effect of Synkavit alone without X-ray; Dittrich and Schmermund (1953) were using an Ehrlich mouse ascites carcinoma. This tumour is known to be radioresistant since a dose of 1250 r causes no increase in dead cells within 48 hours (Klein and Forssberg, 1954). Friedmann and Bailley (1950) used a single intramuscular injection of Synkavit on Jensen rat sarcoma with comparatively small doses of X-ray; Jolles (1952) was studying whole body irradiation of rabbits and guinea-pigs. Cohen and Cohen (1959) used subcutaneous injections of menadione (2-methyl-1:4-naphthoquinone), the corresponding hydroquinone or the tetrasodium salt of the hydroquinone diphosphate (Synkavit), against mammary mouse tumours whose radiocurability at 4200 r was less than 1 per cent. Some comments on this latter paper have already been given (Mitchell, 1959). The other negative results reported by Tarnowski *et al.* (1958) were concerned with carcinoma 63 and sarcoma 180 in mice. Freedlander, Reich, Levitan and French (1958) tested Synkavit as a radiosensitiser of neuroblastoma C-1300 in mice and although the results show obvious sensitization, the compound was not classified as a sensitizer because no tests of the compound alone were given. Negative results against the already-mentioned radioresistant Ehrlich mouse ascites tumour were obtained.

It therefore appears to me, that although most reviews and reports would lead the casual reader to believe that Mitchell's animal radiosensitization results are not reproducible, this view cannot be substantiated.

The small but significant increase in useful life of patients suffering from histologically proved inoperable carcinoma of the bronchus treated by combined intravenous Synkavit and X-ray compared to X-ray and intramuscular Synkavit (which gives no better result than X-ray only) has been proved by clinical trials with random allocation of patients over the past six years (Mitchell, 1955; Mitchell, Simon-Reuss and King, 1959).

I have therefore tried to determine the nature of some of the biochemical processes affected by Synkavit in the hope that an explanation of its radiosensitizing action might follow. An interference with some stage in the biosynthesis of the nucleic acids seemed likely since it was previously shown (Mitchell, 1952) that the antimitotic effect of Synkavit could be altered, and in some cases be completely eliminated, by equimolar amounts of some ribonucleosides and ribonucleotides. The effect of Synkavit on the uptake of labelled formate and glycine into the purines of the acid soluble fraction (ASF), the deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) of the Ehrlich carcinoma growing as an ascites tumour in mice was therefore studied, one of the advantages of this system for this type of experiment being the absence of a variable necrotic area such as is found in solid tumours.

METHODS AND MATERIALS

Mouse ascites tumour.—The Ehrlich carcinoma (hyperdiploid strain of Lettré) was obtained by the kind co-operation of the Chester Beatty Institute in 1948

and has been carried by routine transplantation (0·2 ml.) every 8th day into the peritoneal cavity of our laboratory strain white mice. The experimental animals were used on the 6th or 7th day after implantation depending on the size of the tumour.

^{14}C labelled compounds were obtained from the Radiochemical Centre, Amersham. Most of the counting was done on infinitely thin samples in a Nuclear-Chicago Automatic Windowless Counter operating in an atmosphere of methane in the proportional region. Samples and backgrounds were recorded to a standard error of 5 per cent or less and the specific activities were calculated from optical density measurements on the solutions before they were dried off under a lamp. The molar extinction coefficients were those used previously (Marrian, 1954). Decreasing volumes were dried off and counted until the mean specific activity varied by less than 5 per cent from the extremes.

Ultra-violet absorption measurements were made on a Unicam SP 500 Spectrophotometer, sometimes adapted for use with micro cells.

Estimation of Small Amounts of Synkavit in Solution

Ceric sulphate solution was prepared by shaking 4 g. ceric sulphate with 50 ml. water and 2·8 ml. concentrated sulphuric acid until solution was complete. 50 ml. water was added and the whole clarified by repeated filtration. This solution quantitatively and immediately oxidises Synkavit to 2-methyl-1:4-naphthoquinone (Yamagishi, 1954; Clark, Kirby and Todd, 1958), thereby rendering the molecule soluble in organic solvents. Dilute bromine water has the same action. 0·1 ml. Synkavit solution (optical density 1·44 at 290 mμ.) was added to 10 ml. water and the whole extracted with 2 × 1 ml. pure cyclohexane. The second extract had zero optical density at 250 mμ. (at which wavelength 2-methyl-1:4-naphthoquinone absorbs maximally). 0·1 ml. ceric sulphate solution and 1 ml. cyclohexane were now added and the whole well shaken. The organic phase was seperated off and had an optical density of 0·412 at 250 mμ. Since the molar extinctions of Synkavit at 290 mμ. is 5600 and of the quinone at 250 mμ. is 19,000 this simple extraction represents a recovery of about 79 per cent. Similar estimations are possible using bromine water as the oxidiser, but in this case excess bromine must be removed by a stream of air or nitrogen before extracting with cyclohexane.

Biological experiments.—The experimental mice were divided randomly into two groups. The "treated" mice were injected with 2 mg. Synkavit in 0·1 ml. saline and the "controls" with 0·1 ml. saline. Each mouse was injected with 0·1 ml. of the solution of labelled formate or glycine containing 1 mg. of the precursor and usually about 20 μc. The animals were sacrificed two hours later by breaking their necks.

The acid soluble fraction.—The ascites fluid, at least 8–10 ml. in each batch, was removed by a syringe into an ice cooled centrifuge tube containing a few drops of 2 per cent oxalate solution. An equal volume of cold 0·6 N $HClO_4$ was added and the mixture homogenised in an ice-cooled container (M.S.E. "Atomix" maximum speed for 1 minute). The homogenate and vessel washings were spun at 2000 r.p.m. for 5 minutes (M.S.E. Major centrifuge using precooled containers). The supernatant was filtered into an ice cooled Buchner flask through a pad of Hyflo over a sintered disc which had been well washed with ice water and then ice cold 0·6 N $HClO_4$. The tissue residue was suspended in 5–10 ml.

cold perchloric acid and spun down, the supernatant being filtered as before. This whole process was repeated. The filter pad was finally washed with about 5 ml. cold 0·6 N $HClO_4$. The volume of the acid soluble extract was measured and the solution clarified if necessary by filtering through a paper. A portion was diluted 1/5 with N/10 HCl and the optical density at 260 mμ. (peak) measured. The separation of the purines from this fraction was usually done by evaporation and acid hydrolysis (Marshak and Vogel, 1951) but a method which gives cleaner separation on paper is as follows. The extract was neutralised with aqueous sodium hydroxide solution and 10 ml. 10 per cent silver nitrate added. The precipitate was spun off and heated at 100° with 10 ml. N HCl for 1 hour. The filtered hydrolysate was freed from acid by repeated evaporation *in vacuo* and addition of water, and the final residue dissolved in a little water and run on paper (Whatman 3 mm.) in iso-propanol/HCl (Wyatt, 1951). 10 ml. (about 1/8 of the total) of the neutralised acid soluble fractions (both treated and controls) was acidified with a few drops of concentrated sulphuric acid and gently homogenised with small volumes (1–4 ml.) of pure cyclohexane. The organic phase was sucked off, clarified by spinning, and the optical density at 250 mμ. measured. This extraction was repeated until the cyclohexane had zero optical density: 3 and 6 per cent of the ultraviolet absorbing material was extracted from the control and treated solutions respectively under these conditions. Addition of ceric sulphate solution (or bromine water) caused a further 0·4 and 0·6 per cent to become organic soluble; thus, the injected Synkavit could not have accounted for more than 0·6 per cent of the optical density of the treated acid soluble fraction, but it should be noted that the spectrum of this extracted material did not resemble 2-methyl-1:4-naphthoquinone. Aliquots of the treated and control ASF were diluted with N/10 HCl and the optical density at 260 mμ measured before and after treatment with excess bromine water, the excess being removed by aeration. The optical density of the treated ASF decreased from 7·10 to 4·90, 5·00 and that of the control from 7·15 to 5·68, 5·53 (duplicate estimations).

The tissue residue

The following manipulations were all carried out in the same weighed 6 in. B19 test tube to avoid losses. The tissue residue insoluble in cold dilute perchloric acid, including the small amount from the top of the Hyflo filter pad, was suspended twice in water, enough aqueous sodium hydroxide solution being added to the second suspension to bring the supernatant to neutrality. The insolubles were washed twice with alcohol, once with ether and refluxed for 1 hour with 20 ml. methanol/chloroform (1:1). The solids were then washed with alcohol, ether and dried *in vacuo* and the total weight noted. The dried solids were well mixed with a glass rod to ensure homogeneity.

Separation of RNA and DNA purines and counting of the samples was carried out as previously described (Marrian, 1954; Marrian, Hughes and Werba, 1956).

Estimations of DNA were carried out in duplicate on 20–30 mg. samples of the tissue residue as described by Burton (1956).

RESULTS AND DISCUSSION

The results of these experiments are given in Tables I, II and III as the ratio between the specific activity of the purines in the Synkavit treated and in the

control mice (Relative Specific Activity). The variability of the results necessitated many repetitions and, indeed, the final figures are the means of 22 experiments where formate was the precursor; the addition of 5 experiments where glycine was used did not alter the figures appreciably. Occasionally, some of the results were so widely different from the means that some error either in manipulation or in the metabolic state of either the control or treated mice had occurred. Such results were eliminated from the final calculations by Chauvenet's criterion (Palmer, 1912, quoted and discussed by Calvin *et al.*, 1949); the number of results deleted and their value are given in the tables. The arithmetical means and standard deviations are given, and the means have been recalculated using logarithmic values since biological experiments are frequently better so expressed. In the present series however, there is no significant difference between the results by either calculation, but the logarithmic calculations require fewer deletions by Chauvenet's criterion. The results left little doubt that one effect of the injected Synkavit was to inhibit the synthesis of RNA purines from low molecular weight precursors without affecting the synthesis of either DNA purines or the acid soluble purine nucleotides. This suggested that the mode of action was to interfere with the polymerisation of the acid soluble nucleotides to RNA and it would follow that an accumulation of acid soluble nucleotides should occur. This was indeed shown to be the case by comparing the amount of acid soluble ultraviolet absorbing material per unit amount of DNA in the corresponding dry tissues residues in each case. The results of an experiment where 8 mice were divided randomly and treated as before is given in Table IV. The increase shown in Table IV in the amount of acid soluble nucleotides per unit amount of DNA in the Synkavit treated mice compared to the controls is not only in agreement with the postulated block between the acid soluble fraction and the RNA but the extent of the increase (23 per cent) is in very close agreement with the amount by which the activity of the treated RNA purines have fallen below the control RNA purines in the amount of precursor utilised (24 and 20 per cent for adenine and guanine respectively).

TABLE I.—*Acid Soluble Purines—Relative Specific Activities (R.S.A.)*

22 Experiments with Formate as Precursor

	Number of observations	Observations deleted	R.S.A. Arithmetical	R.S.A. Logarithmic
Adenine	22	Ar. 0·34 Lg. 0·34	1·01±0·06	$0·97\left\{\begin{matrix}+0·06\\-0·04\end{matrix}\right.$
Guanine	18	Ar. 1·75, 1·94, 6·30 Lg. 6·30	1·09±0·07	$1·12\left\{\begin{matrix}+0·11\\-0·10\end{matrix}\right.$

22 Experiments with Formate and 5 with Glycine as Precursor

	Number of observations	Observations deleted	R.S.A. Arithmetical	R.S.A. Logarithmic
Adenine	27	Ar. 0·34, 0·29 Lg. 0·34, 0·29	0·99±0·05	0·95±0·05
Guanine	22	Ar. 1·75, 1·94, 6·30 Lg. 6·30	1·05±0·07	$1·07\left\{\begin{matrix}+0·08\\-0·07\end{matrix}\right.$

TABLE II.—*Deoxyribonucleic Acid Purines—Relative Specific Activities (R.S.A.)*

22 Experiments with Formate as Precursor

	Number of observations	Observations deleted	R.S.A. Arithmetical	R.S.A. Logarithmic
Adenine	21	Ar. 3·6, 1·64, 2·52, 1·91 Lg. 3·6	0·90±0·06	0·98 $^{+0·11}_{-0·08}$
Guanine	21	None	1·00±0·06	0·95 $^{+0·05}_{-0·07}$

22 Experiments with Formate and 5 with Glycine as Precursor

	Number of observations	Observations deleted	R.S.A. Arithmetical	R.S.A. Logarithmic
Adenine	24	Ar. 3·6, 1·64, 2·52, 1·91 Lg. 3·6	0·87±0·05	0·93 $^{+0·11}_{-0·07}$
Guanine	24	None	0·98±0·06	0·94 $^{+0·05}_{-0·06}$

TABLE III.—*Ribonucleic Acid Purines—Relative Specific Activities (R.S.A.)*

22 Experiments with Formate as Precursor

	Number of observations	Observations deleted	R.S.A. Arithmetical	R.S.A. Logarithmic
Adenine	20	Ar. 5·0, 1·8, 6·3, 1·8 Lg. 5·0, 6·3	0·74±0·07	0·74 $^{+0·09}_{-0·08}$
Guanine	20	Ar. 1·43, 1·78 Lg. 1·78	0·81±0·06	0·83±0·07

22 Experiments with Formate and 5 with Glycine as percursor

	Number of observations	Observations deleted	R.S.A. Arithmetical	R.S.A. Logarithmic
Adenine	25	Ar. 5·0, 1·8, 6·3, 1·8 Lg. 5·0, 6·3	0·76±0·06	0·76±0·07
Guanine	25	Ar. 1·43, 1·78 Lg. 1·78, 0·19	0·80±0·06	0·78±0·06

TABLE IV.—*Accumulation of Acid Soluble Nucleotides after Synkavit Treatment.*

	Treated	Controls
Volume of ascites fluid	20 ml.	25 ml.
Total ASF by O.D. at 260 mμ.	596 units	604 units
Total DNA in the tissue residues by O.D. at 600 mμ. (Duplicate estimation)	250, 252 units	296, 292 units
Ratio DNA/ASF	2·38	2·06
Ratios from 3 earlier experiments	2·0	1·6
	1·8	1·7
	2·1	1·5
Mean	2·1±0·1	1·7±0·1

It was, of course, necessary to show that the increase in acid soluble ultraviolet absorbing material was not due merely to the presence of the injected Synkavit or some water soluble derivative. However, ceric sulphate or bromine water estimations for the presence of Synkavit showed that the amount of Syn-

kavit which could have been present was insignificantly small. The action of dilute bromine water on mixed purine and pyrimidine derivatives is to make the latter transparent to ultraviolet light, thus allowing a crude differential analysis. The ratio of pyrimidine to purines thus found in the control ASF was 0·28, while that in the treated was 0·44 suggesting that the increase in the nucleotides was not uniform, the pyrimidine nucleotides increasing in concentration relatively more than the purines.

The action of Synkavit as a radiosensitizer may therefore involve interference with RNA synthesis, while the X-ray is interfering with DNA synthesis, and this two-pronged attack on the cell's functions may account for the more than additive effect of combined treatments.

Some avenues for future work are suggested by these results. It will be interesting to see whether Synkavit will inhibit polynucleotide phosphorylase, the enzyme which Grunberg-Monago, Ortiz and Ochoa (1955) have shown to polymerise ribonucleoside diphosphates to RNA like material. Furthermore, if it is the two sided approach of combination therapy which is involved, then Synkavit may increase the desired effects of therapy by alkylating agents allowing, perhaps, remissions of acute leukaemia with less toxic side effects. And Actinomycin D, which appears to sensitize human skin to irradiation (Kingsley-Pillers—personal observations) may show this effect through an interference with RNA synthesis.

SUMMARY

The effect of tetrasodium 2-methyl-1:4-naphthohydroquinone diphosphate (Synkavit) on the uptake of labelled formate or glycine into the purines of mouse ascites tumour has been studied over a 2-hour period. The compound does not alter the uptake of the precursor into the purines of the acid soluble nucleotides or of the deoxyribonucleic acid, but the uptake into the ribonucleic acid purines is reduced by 20–25 per cent compared to the controls. This inhibition is accompanied by an increase in 23 per cent of the total ultraviolet absorption of the acid soluble fraction which could not be accounted for by the presence of the injected compound in this fraction. The pyrimidines in this fraction increased in concentration relative to the purines.

The implications of these observations are discussed.

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REFERENCES

Burton, K.—(1956) *Biochem. J.*, **62**, 315.
Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M. and Yankwich, P. F.—(1949) " Isotopic Carbon ", New York (John Wiley and Sons Inc.), p. 290.
Clark, V. M., Kirby, G. W. and Todd, A. R.—(1958) *Nature*, **181**, 1650.
Cohen, L. and Cohen, A.—(1959) *Brit. J. Radiol.*, **32**, 18.

DITTRICH, W. AND SCHMERMUND, H. J.—(1953) *Strahlentherapie*, **90**, 88.
FREEDLANDER, B. L., REICH, S., LEVITAN, J. AND FRENCH, A.—(1958) *Cancer Res.*, **18** No. 8, Pt. 2), 447.
FRIEDMANN, E. AND BAILLEY, N. J. T.—(1950) *Biochim. biophys. Acta*, **6**, 274.
GELLHORN, A. AND GAGLIANO. T.—(1950) *Brit. J. Cancer*, **4**, 103.
GRUNBERG-MANAGO, M., ORTIZ, P. J. AND OCHOA, S.—(1955) *Science*, **122**, 907.
JOLLES, B.—(1952) *Rep. Brit. Emp. Cancer Campgn*, **30**, 324.
KLEIN, E. AND FORSSBERG, A.—(1954) *Exp. Cell. Res.*, **6**, 211.
MARRIAN, D. H.—(1954) *Biochim. biophys. Acta*, **13**, 282.
Idem, HUGHES, A. F. W. AND WERBA, S. M.—(1956) *Ibid.*, **19**, 318.
MARSHAK, A. AND VOGEL, H. J.—(1951) *J. biol. Chem.*, **189**, 597.
MCKELVIE, A. D.—(1959) *Nature*, **183**, 1194.
MITCHELL, J. S.—(1952) *Rep. Brit. Emp. Cancer Campgn*, **30**, 238.—(1953) *Acta Radiol., Stockh.* (Congress Volume) Suppl. **116**, 431.—(1955) "Radiobiology Symposium" (Liege) 1954. London. (Butterworths Scientific Publications) p. 170.—(1959) *Brit. J. Radiol.*, **32**, 214.
Idem AND SIMON-REUSS, I.—(1947) *Nature*, **160**, 98.—(1952*a*) *Brit. J. Cancer*, **6**, 305.—(1952*b*) *Ibid.*, **6**, 317.
Iidem AND KING, E. A.—(1959) *Acta Un. int. Cancr.* (In press.)
PALMER, A. DE F.—(1912) "Theory of Measurements" New York., (McGraw Hill Book Co.), p. 127 *et seq.*
TARNOWSKI, G. S., BANE, H. N., CONRAD, J., NICKSON, J. J., STOCK, C. C. AND SUGIURA, K.—(1958) *Cancer Res.*, **18**, (No. 8, Part 2), 225.
WYATT, G. R.—(1951) *Biochem. J.*, **48**, 584.
YAMAGISHI, M.—(1954) *Rep. Takeda res. Lab.*, **13**. 25.

BIOLOGICAL EFFECT OF 1 : 6-DIMETHANE-SULPHONYL-D-MANNITOL IN ANIMAL EXPERIMENTS

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A COMPOUND far less toxic than nitrogen mustard is obtained by linking the chloroethylamino radical to a sugar molecule, particularly a mannitol molecule (BCM, Degranol, 1:6-bis) (β-chloroethylamino)-1:6-desoxy-D-mannitol dihydrochloride) (Vargha, 1955; Vargha, Toldy, Fehér and Lendvai, 1957; Kellner and Németh, 1956). Hungarian workers have long ago entertained the idea that if instead of the chloroethylamino radical the sulphonoxyd radical is bound to mannitol the result must be a compound of lesser toxicity than Myleran. At the London Congress of 1958, and later in a preliminary communication, Haddow, Timmis and Brown (1958) reported the preparation of this compound and its effects on the Walker rat carcinoma-256, the "August" rat carcinoma, and a radiation-induced rat leukaemia. In work based on the earlier Hungarian conception and running parallel with the efforts of the English authors, Vargha and Kuszmann (1959) also prepared 1:6-dimethane-sulphonyl-D-mannitol (hereafter referred to as MM), but by a different route. Their compound was submitted to us in September 1958 to determine its biological properties.

Our findings to date show MM to possess excellent toxicological and pharmacological properties. It exerts a marked effect on the blood picture, inhibits the growth of various different transplantable tumours, heavily damaging the tumour cells and altering the tissue structure in its entirety and gives rise to distinct morphological changes in organs (bone marrow, duodenum, spleen, testes).

Unlike Myleran, MM is readily soluble in water. This explains why the two compounds differ in biological properties. It is much less toxic than Myleran, and this is a quality of deep importance (Table I). Soon after it is dissolved in water the pH signifies acidity when, on the evidence of data obtained in tissue culture, its destructive effect on tumour cells decreases, though it does not cease altogether. Minimum lethal doses of it kill experimental animals in four or five days, but if administered in fractions death occurs as late as in 14 to 20 days. Owing to the marked cumulative effect of the drug, tests to determine the LD_{50} must be carried on for at least four weeks, not infrequently for 60 days, occasionally for a still longer period of time.

In animals which died from acute MM poisoning, autopsy showed the intestines to be inflated with fluid, the mucous membrane to be hyperaemic, with extensive haemorrhages on both the mucosa and serosa; the spleen was remarkably wasted; the lungs were oedematous, with haemorrhagic spots in them; transudate accumulated in the body cavities; the bone marrow was vividly red, and after a more than usually large dose, slightly jellied.

Certain observations have been made which, though requiring further studies and detailed confirmation, show that some considerable time after the introduction

of a larger dose of MM grave lesions may arise in the organism (abscesses on the skin, decubituses on the extremities, emaciation, exsiccosis, pneumonia, tuberculosis, etc.), with tumour-formation in some cases (leukemia, sarcoma, hypophysis tumour, etc.).

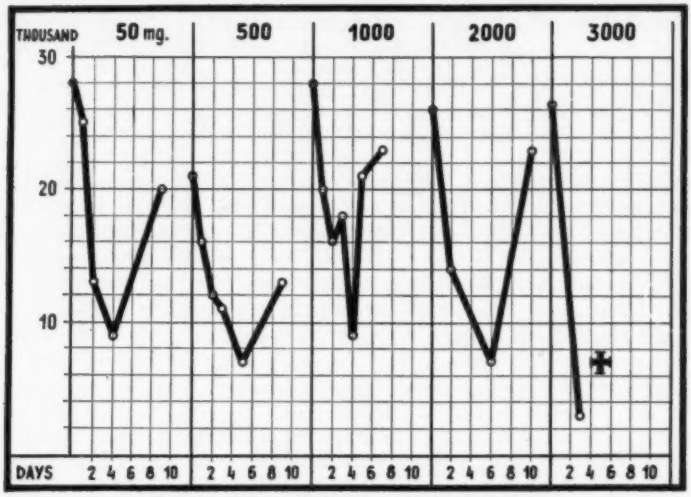


FIG. 1.—Effect of MM on the W.B.C. of rats.

TABLE I.—*Pharmacological Data Determined for MM (Observation period : 30 days)*

		mg./kg.
LD_{100}	For the mouse[1]	5·000 i.p.
	For the rat[2]	4·000 i.p.
	For the rabbit	3·000 oral
LD^{3}_{50}	For the mouse	4·300 i.p.
	For the rat	2·900 i.p.
Maximum tolerated dose[3]	For the mouse	2·000 i.p.
	For the rat	1·500 i.v. and i.p.
Therapeutic dose[4]	For Ehrlich ascites tumour	200 i.p.
	For resistant solid tumour (Crocker)	500 i.p.
	For sensitive solid tumour (Yoshida)	100–200 i.v. and i.p.
Minimum effective dose (determined by changes in the blood picture of the rat)	—	50 i.v.
Cumulative action	—	1×1500 : 0/10[5] 15× 100 : 5/10[5] 1×1000 : 105[6] 5× 200 : 92[6]

[1] C_3 strain.
[2] Chester Beatty strain.
[3] Given on one occasion.
[4] Given for seven consecutive days.
[5] Mortality of mice.
[6] Per cent body weight change on the 4th day (rat).

In 24 to 48 hours following the injection of a relatively small dose, the first to decrease in number were the neutrophil granulocytes in the blood stream : one tenth of the maximum tolerated dose, or 50 mg./kg., reduced their number by 20 to 50 per cent in the rat and the rabbit. In the leukocyte count, on the action of this dose the maximum decrease usually presented itself on the fourth day (Fig. 1), and was soon followed by a compensating leukocytosis lasting for about three weeks (Table II). The lymphocytes were left unaffected by a similar dose ;

TABLE II.—*Changes in the Blood Picture of the Rabbit due to the Action of MM.*

	100 mg./kg. i.v.				200 i.v.				300 i.v.				500 oral			
Days	W.B.C. 10^3	Ne 10^3	Ly 10^3	R.B.C. 10^6	W.B.C. 10^3	N 10^3	L 10^3	R.B.C. 10^6	W.B.C. 10^3	N 10^3	L 10^3	R.B.C. 10^6	W.B.C. 10^3	N 10^3	L 10^3	R.B.C. 10^6
Before treatment	5·1	*1·2*	3·9	5·0	4·6	0·9	3·7	5·6	4·6	*1·4*	3·2	..	6·3	*1·3*	5·0	5·6
1	4·2	1·0	3·2	5·5	6·6	1·8	4·8	5·2	4·4	1·0	3·4	5·6	4·9	0·9	4·0	5·4
2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	3·8	0·7	3·1	4·7	5·2	1·6	3·6	5·8	3·8	0·8	3·0	—	—	—	—	—
4	—	—	—	—	—	—	—	—	—	—	—	—	3·8	0·6	3·2	5·2
5	3·4	0·5	2·9	5·1	4·6	0·5	4·1	5·8	6·6	0·4	6·2	4·7	—	—	—	—
6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
7	—	—	—	—	—	—	—	—	—	—	—	—	5·9	*0·3*	5·6	3·8
9	3·9	0·4	3·5	—	4·3	0·5	3·8	—	3·8	*0·15*	3·65	—	—	—	—	—
11	—	—	—	—	—	—	—	—	—	—	—	—	9·6	1·4	8·2	4·2
12	3·7	*0·2*	3·5	4·0	2·3	0·3	2·0	5·4	6·2	3·8	2·4	4·6	—	—	—	—
14	—	—	—	—	—	—	—	—	—	—	—	—	9·3	1·4	7·9	5·0
16	2·5	0·7	1·8	4·6	7·2	1·6	5·6	5·2	4·8	0·5	4·3	5·0	—	—	—	—
18	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
21	3·8	0·6	3·2	4·2	5·9	0·9	5·0	4·0	6·8	1·4	5·4	5·2	—	—	—	—
29	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
50	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
56	—	—	—	—	—	—	—	—	—	—	—	—	2·8	—	—	5·6
														Exitus		
74	—	—	—	—	—	—	—	—	4·2	—	—	5·2				
										Exitus						

	1000 oral				2000 oral				3000 oral				5×200 i.v.			
Days	W.B.C. 10^3	N 10^3	L 10^3	R.B.C. 10^6	W.B.C. 10^3	N 10^3	L 10^3	R.B.C. 10^6	W.B.C. 10^3	N 10^3	L 10^3	R.B.C. 10^6	W.B.C. 10^3	N 10^3	L 10^3	R.B.C 10^6
Before treatment	*9·6*	6·7	2·9	5·2	*12·0*	4·8	7·2	5·2	*5·7*	0·8	4·9	5·6	*6·8*	*2·4*	4·4	5·2
1	10·0	7·7	2·3	5·6	9·9	4·8	5·1	5·6	5·5	2·2	3·3	—	2·1	0·8	1·3	—
2	7·7	6·4	1·3	5·0	—	—	—	—	4·0	1·8	2·2	5·2	2·1	0·5	1·6	—
3	4·2	1·5	2·7	4·2	—	—	—	—	*2·1*	*0·01*	2·09	4·8	2·3	0·5	1·8	—
4	*4·2*	*1·1*	3·1	—	*2·8*	*0·05*	2·75	5·0	—	—	—	—	—	—	—	—
5	—	—	—	—	—	—	—	—		Exitus			0·9	0·1	0·8	5·2
6	—	—	—	—	—	—	—	—	—	—	—	—	*1·7*	*0·2*	1·5	4·8
7	11·0	3·5	7·5	—	11·3	5·65	5·65	4·6	—	—	—	—	4·8	2·3	2·5	4·2
9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
11	12·7	8·9	3·8	4·4	13·3	7·3	6·0	5·2	—	—	—	—	—	—	—	—
12	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
14	14·4	10·8	3·6	—	9·5	4·75	4·75	5·0	—	—	—	—	—	—	—	—
16	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
18	10·7	8·3	2·4	—	—	—	—	—	—	—	—	—	—	—	—	—
21	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
29	—	—	—	—		Exitus			—	—	—	—	—	—	—	—
50	18·0	—	—	8·6	—	—	—	—	—	—	—	—	—	—	—	—
		Exitus														
56	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
74	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

for them to decrease in number a dose was required which was capable of reducing the number of neutrophils by more than 50 per cent; even then, a relative lymphocytosis sometimes developed owing to the high degree of neutropenia. The thrombocyte count remained the same unless the drug was introduced in a quantity approaching the median lethal dose. Erythropoiesis was left intact even by that dose, but a lethal dose gave rise to haematologic changes resembling grave agranulocytosis, and red blood corpuscles began to be destroyed upon its action.

When the doses were administered in fractions, the changes in the blood picture were much more conspicuous.

In the bone marrow, smaller doses induced no particularly remarkable lesions in the mouse and the rabbit, although the bone marrow of the latter was slightly more sensitive. Doses of the drug approaching the maximally tolerated level produced significant reductions in the number of myeloid elements, but left the erythropoietic cells, the megakaryocytes and the reticular elements undamaged. Quantities in excess of the maximum tolerated dose gave rise to grave changes in the bone marrow, causing pycnotic disintegration of large numbers of cells and reducing the number of cells of intact structure; the capillaries among them appeared dilated, and the remaining megakaryocytes become all the more conspicuous. Treatment even with large doses given for a long period of time failed to reveal changes pointing to serious lesions in erythropoiesis.

The tumour-inhibiting action of the drug is apparently selective: it differed in fair degree in the six tumour types involved in our tests (Yoshida rat sarcoma, Guérin rat carcinoma, Ehrlich adenocarcinoma, Crocker sarcoma 180 in mice and the 36th passage of ENG spontaneous sarcoma in mice) (Table III). This obviously makes it desirable to test the compound against a wider spectrum of tumours for its growth-inhibiting activity.

TABLE III.—*Growth Inhibiting Action of MM on Transplanted Tumours*

Type of tumours	Number of animals	Dose (mg./kg./day)	Percentage inhibition
Yoshida solid	5	5×500 i.v. Killed on 6th day	77
Guérin	10	9×500 i.v. Killed on 10th day	95
Crocker	10	10×500 i.p. Killed on 11th day	52
ENG mouse-sarcoma	10	5×500 i.p. Killed on 6th day	87
Ehrlich ascites	10	6×200 i.p. Killed on 8th day	36
Ehrlich subcutaneous	10	6×500 i.p. Killed on 8th day	30

Generally speaking, MM has a more marked inhibitory effect on transplanted tumours of the rat than on those of the mouse. A fact meriting special attention is that on the Guérin tumour, known to be fairly resistant to most drugs of therapeutic effect, as much as 90 per cent inhibition of growth was seen, and on the intravenous administration of a very large quantity (9–10 × 500 mg./kg.), even recovery was attained. A much smaller amount of the drug sufficed to bring about recovery in Yoshida tumour.

The question of how MM affects survival has not yet been fully investigated.

Nevertheless, on the strength of experience that has been gained so far, it seems safe to say that, given in suitable doses, the compound is capable not only of retarding growth, but also of protracting survival time.

In tissue culture, MM was observed to have growth-inhibitory and destructive effects on fibroblasts of chick-embryo hearts and fresh Crocker sarcoma and HeLa cells. Cultures incubated for 24 hours were treated with solutions of various concentrations of the drug, and after an additional 24 hours the extent of damage was evaluated, and expressed by ±, +, ++, or +++. After adding sodium bicarbonate, to bring the pH from acid to neutral, a 1000 γ/ml. dose was enough to elicit a very marked effect, while double this dose was found to destroy all the cultures. With a solution which had not been treated to prevent a fall in pH (pH 5·8), a dose of as much as 5000 γ/ml. was required for the drug to exercise its maximum effect (Table IV). It is remarkable how nearly the doses found efficaceous in explants, agree with those established in our experiments *in vivo*. This seems to indicate that explantation might be of value in determining the doses.

TABLE IV.—*Effect of MM on Tissue Culture*

	Fibroblast		Crocker		HeLa	
Gamma/ml.	1	2	1	2	1	2
250	..	+	..	+	..	..
500	+	+	+	+	+	..
1000	+++	+	+++	+(+)	++	..
2000	+++	..	+++	..	+++	..
5000	..	++(+)	..	+++	..	..

1. pH : 7·0.
2. pH : 5·8.

In Ehrlich ascites tumour, MM—like chemotherapeutic agents in general—produced a reduction in the cell count, an increase in the number of eosinophils, and caused changes in cellular morphology and acridine-orange uptake. It is interesting to note that whereas after the introduction of most agents (chloroethylamino derivatives, antimetabolites, etc.) acridine-orange uptake decreases, it invariably increased upon the action of MM, quite like upon that of Myleran (Table V).

TABLE V.—*Changes in Number and Staining Capacity of Ehrlich Ascites Cells Following Treatment of MM*

	Controls (%)	MM 500 mg./kg. intraperitoneal				
		6	24	48	72	96
		Hours				
Number of cells*	100	51	36	39	30	16
Cells staining with eosin†	5	8	16	37	29	40
Acridin orange-uptake‡	100	89	223	273	219	—

* 10^6/ml. = 100 per cent.
† In percentage of total cells.
‡ 10^{-3} gamma/cell = 100 per cent.

Similarly to Degranol and several other chemotherapeutic drugs, MM prevents many of the intravenously introduced tumour cells from taking, and inhibits

metastasis production. Twenty-four hours after the intravenous introduction of ten million Guérin carcinoma cells, each of a group of five animals was given into the veins seven consecutive daily doses of 500 mg. of MM, and another group was left untreated to serve as controls. All were sacrificed on the 24th day following transplantation. Autopsy revealed extensive metastases in the lungs, adrenals, and lymph nodes of the controls, but none in four of the treated experimentals, while in the fifth, only a few metastases were found, restricted to lymph nodes (Fig. 2). We regard this protective action of MM as significant since on the evidence of our earlier findings (Lapis and Németh, 1956) Myleran does not possess it; in addition, it may prove to be of value before and after surgical treatment of tumours.

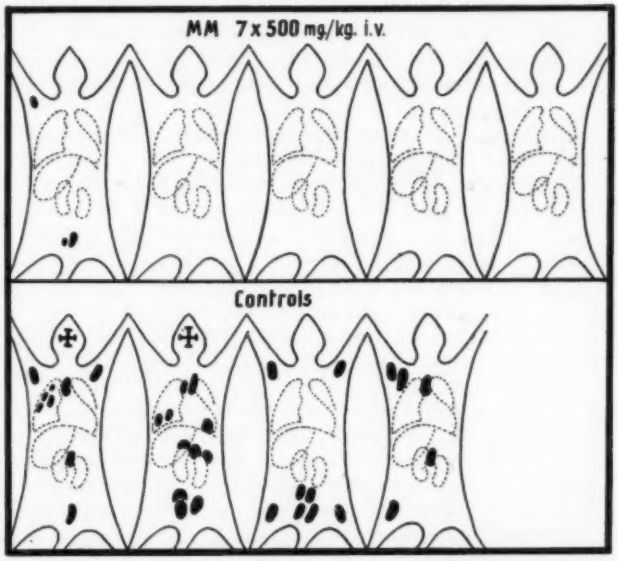


FIG. 2.—Effect of MM on the metastasis of intravenously induced Guérin carcinoma as observed on the 20th day.

In our studies of the dynamics of the morphological changes caused by MM, Guérin tumours were chosen: first, because the cells in their viable parts are fairly uniform; secondly, because in Guérin tumours of roughly equal size, the proportion of the area of the viable parts to that of the circumscribed necrotic parts of moderate extent, is largely the same; and thirdly, because MM powerfully inhibits the growth of this type of tumour. About ten days after transplantation, animals bearing tumours the size of a small walnut were selected and given intravenously a single 2000 mg./kg. dose of MM. These were killed one by one, 1, 3, 6, 10, and 12 hours, 1, 2, 3, 4, and 5 days, after injection. Sections were prepared of the tumours at the largest diameter, and of the more important organs. The morphological changes seen were essentially the same as those produced by the other alkylating agents (nitrogen mustard, Degranol, Sarcolysin, Mitomen, etc.). We describe them in the following (Table VI).

TABLE VI.—*Cytological Changes Upon the Action of MM*

	Control	Found in tumour: Hours after administration					
		10	24	48	72	92	120
Intact cells	6·7	0·8	0·2	0·1	—	—	—
Mitosis, anomalous or disintegrating	1·2	7·5	15	26·7	22·4	6·4	9·6
Bi- and multinuclear cells	3·6	3·8	17·7	40·4	78·6	47·5	49·3
Necrosis	+++	++	++	++	+++	++++	++++
Assayed in duodenum							
Decrease in number of mitotic cells	..	++	+++	+++	+++	++	—
Anomalous mitotic cells	+	+++	—	—	—	+++	+
Disintegrating mitotic cells	—	++	+++	++	+	—	—
Resting cells	+	+	+	++	+++	+++	++
Giant cells	..	..	..	..	+	+	..

\+ From sporadic to few.
++ From few to about half.
+++ From half to many.
++++ Between many and almost all.

After 12 hours, only cells in the process of mitosis are affected : the chromatin is loosened in structure or reduced to powder, the chromosomes have drifted away and broken into fragments, a bridge is formed between the two mitotic spindles, etc. After one day, but more conspicuously so after two days, anomalous mitoses increase in number, pycnotic nuclear destruction sets in, and binuclear and multinuclear cells suddenly appear. Thereafter, anomalous mitoses decrease in number, and pycnotic disintegration becomes so extensive within the viable parts as to loosen the structure of the tumour. The central necrotic areas keep growing in size until on the 4th or 5th day the entire tumour is but a necrotic mass, with only a very thin envelope of retained tumour tissue around it, in which most of the tumour cells, showing two or more nuclei are seen surrounded by an increasing number of connective tissue fibres (Fig. 3 and 4).

In the duodenum, mitoses were no longer seen after 10 hours, but pycnotic nuclei were encountered in very large numbers. By the third day, the villi were broadened and flattened, their connective tissue was extensively infiltrated by globular cells, there was cystic dilatation of the glands, and the epithelial cells were flattened or turned into multinuclear giant cells. By the fifth day, hardly any traces of these changes were seen (Fig. 5).

Morphological changes of the above description—enlargement of the necrotic areas, mitotic anomalies, and pycnotic disintegration of tumour cells—are seen in Crocker 180 mouse carcinoma and ENG mouse sarcoma, but they are of a much milder nature and require considerably larger doses to arise (3 to 6000 mg./kg.).

SUMMARY

The compound prepared by linking the sulphonoxyd radical with mannitol is readily soluble in water, much less toxic than Myleran, and possesses superior biological qualities.

In tissue culture, on the use of a neutral medium it exercises a powerful destructive effect on fibroblasts, and Crocker and HeLa cells.

It is capable of potentially damaging Ehrlich ascites cells, as is evidenced by a decrease in their number upon its action, and an increase in their capacity to stain with eosin and acridine orange.

One tenth of the maximally tolerated dose of the compound suffices to give rise to marked changes in the blood picture; the granulocytes in the blood stream and the myeloid elements of the bone marrow suffer most. Lymphocytes are left unaffected by doses of less than at least a 50 per cent neutrophil-depressing activity. Toxic doses are required to do damage to thrombocytes. The least effect the compound has is that on the erythropoietic system.

In six different types of rat and mouse tumours its growth-inhibiting action was found to range from 30 to 90 per cent. Recoveries were attained with it in Yoshida and Guérin tumours. Unlike Myleran, it inhibited metastatic diffusion to the organs after intravenous transplantation.

Its destructive effect on tumour cells, studied chiefly in Guérin rat carcinoma, is similar to but more lasting than that of the other alkylating agents.

Preliminary data obtained in as yet incomplete investigations indicate that the compound may possess a cumulative effect and a carcinogenic action which manifests itself early (mouse leukaemias, endotheliomas associated with ascites, etc.).

Our thanks are due to Dr. Martha Schmidt and Dr. Ibolya Fogarassy for diverse laboratory tests carried out with rabbit blood; to Dr. J. Sugár and Dr. Éva Gáti for investigations into the straining capacity of ascites tumours; to I. Pályi and Emily Gréczy for tissue culture experiments; and to G. Gálffy for his assistance in our toxicological and biological studies.

REFERENCES

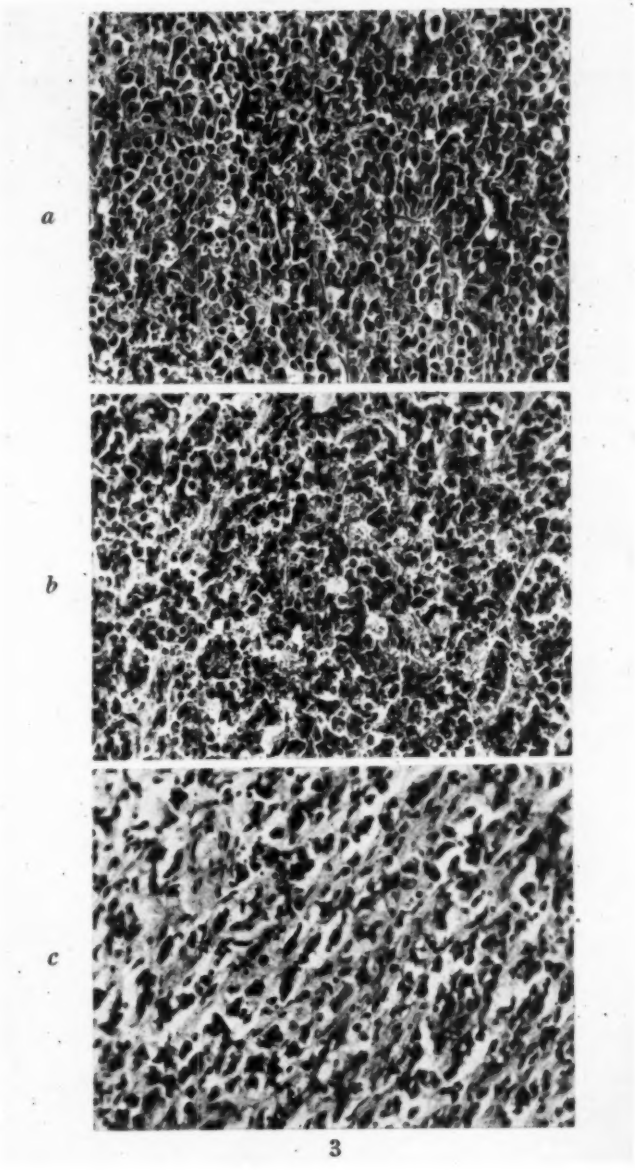
HADDOW, A., TIMMIS, G. M. AND BROWN, S. S.—(1958) *Nature*, **182**, 1164.
KELLNER, B. AND NÉMETH, L.—(1956) *Z. Krebsforsch.*, **61**, 165.
LAPIS, K. AND NÉMETH, L.—(1956) *Brit. J. Cancer*, **10**, 719.
VARGHA, L.—(1955) *Naturwissenschaften*, **42**, 582.
Idem AND KUSZMANN, J.—(1959) *Ibid.*, **2**, 84.
Idem, TOLDY, L., FEHÉR, Ö. AND LENDVAI, S.—(1957) *J. chem. Soc.*, 805.

EXPLANATION OF PLATES

FIG. 3.—Morphological changes in Guérin rat carcinoma due to the action of MM:
(*a*) Controls. Viable portion. × 200.
(*b*) Picture seen 60 hours after injection of a single dose of 2000 mg./kg. dose. Note extensive pycnotic nuclear disintegration, loosened structure, and the presence of cells with two and more nuclei. × 200.
(*c*) Picture seen 120 hours after injection of the same dose. Note that most of the cells are now bi- or multinuclear and that there are now more connective tissue fibres. × 200.

FIG. 4.—(*a*) Great number of anomalous mitotic forms and disintegrating cells. Chromatin in resting cells clumped or of a lytic light colour. (Identical with Fig. 3*b* but × 1620.)
(*b*) Giant cells and increased amount of connective tissue. (Identical with Fig. 3*c* but × 1620.)

FIG. 5.—Pictures of the duodenum seen after administration of a single dose of 2000 mg./kg.
(*a*) Ten hours after injection: mitoses no longer visible; numerous pycnotic nuclei and chromatin globules formed from nuclear fragments; most cells at rest turned light in colour; great number of goblet cells. × 220.
(*b*) Seventy-two hours after injection: duodenal mucous membrane very thin; villi broadened; glands dilated cyst like; epithelial lining flattened; infiltration of globular cells in connective tissue extending as far as the submucosa.



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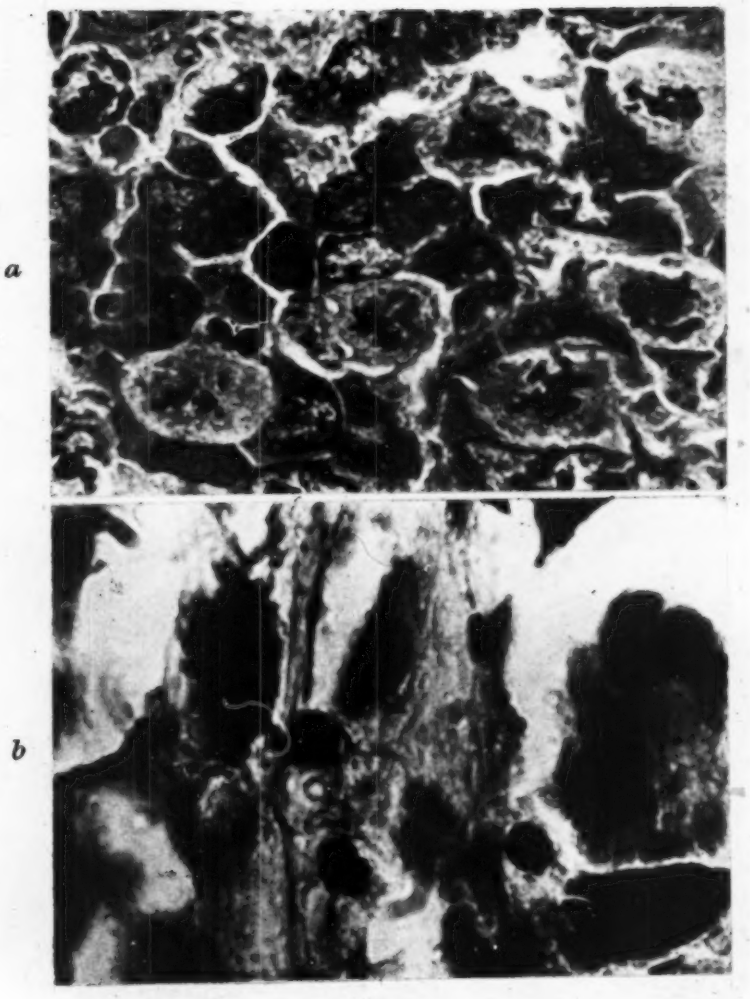

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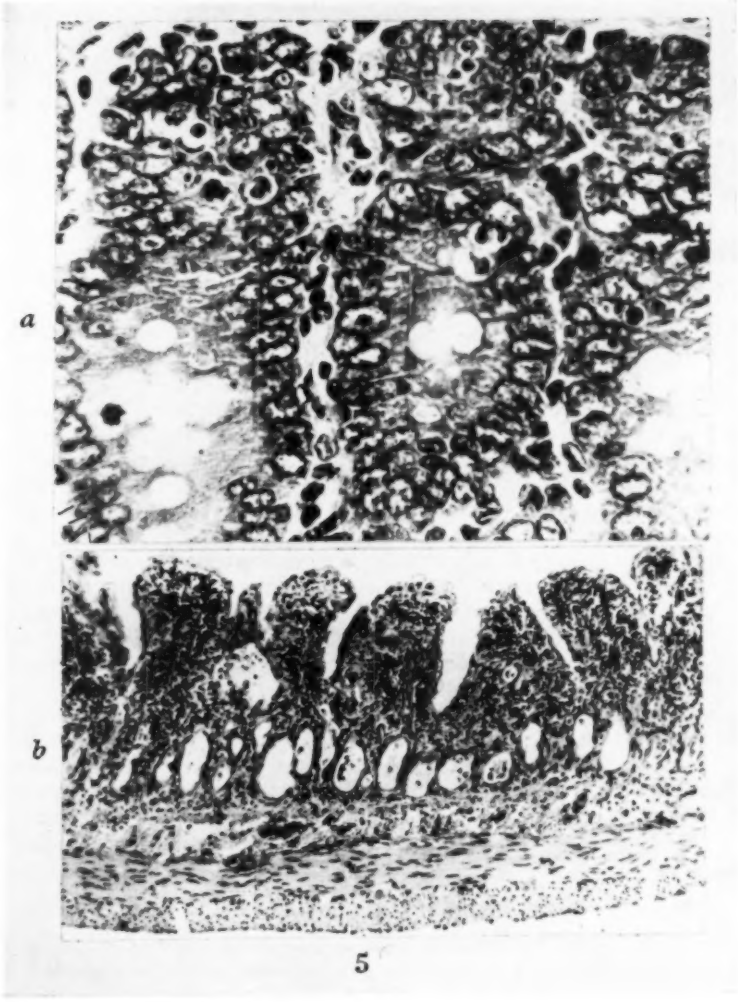

5

SERIAL IRRADIATION OF MOUSE TUMOURS: CHANGES IN RADIOSENSITIVITY

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INCREASED radioresistance of a tumour after a number of sublethal irradiation doses have been administered, has been frequently reported clinically (Paterson, 1949; Cade, 1948; Windeyer, 1954). The action of the initial irradiation in selecting the more radioresistant cells from a heterologous population, inducing radioresistant mutants and the production of alterations in the tumour bed, have been proposed as explanations of acquired radioresistance (Conger and Luippold, 1957).

Experimental evidence of a direct selective or mutational effect on the tumour cells, resulting in a condition of increased radioresistance, has been contradictory. Irradiation lines of transplantable animal tumours, obtained by serial irradiations and transplantations into fresh animals, have been frequently employed to evaluate these changes. Hill, Morton and Witherbee (1919) employing mouse adenocarcinoma 63, Russ (1924), Mottram (1932) and Snellman (1935) on Jensen's rat sarcoma, and more recently Dittrich, Hohne and Schubert (1956) and Lettré (1956) on Ehrlich mouse carcinoma have claimed positive evidence of an increase in radioresistance. Negative evidence has been reported by Bagg (1938) on sarcoma 180, Montgomery and Warren (1953) and Nice (1957) on transplantable mouse lymphosarcomata, and Conger and Luippold (1957) and Baillif (1958) on Ehrlich mouse carcinoma.

The present study was undertaken to examine the effect on radiosensitivity of serial irradiations on transplantable mouse tumours, where the total administered dose reached a value many times the lethal level. The experiments cited above were conducted on well established tumours, usually of a non strain specific type. In this study two tumours arising in an inbred mouse strain were used as experimental material together with a homotransplant—sarcoma 37.

MATERIALS AND METHODS

All experiments were conducted on RIII strain inbred mice. Sarcoma 37 was chosen for study owing to the regularity of its growth rate and absence of spontaneous regressions. The two homologous tumours were a spindle-celled sarcoma (SP1), induced in an inbred RIII strain mouse by subcutaneous injection of 3 · 4-benzpyrene colloid and passaged 6 times before the commencement of these experiments, and a spontaneous mammary adenocarcinoma (MV212) arising in the same mouse strain and previously passaged 17 times. All tumours were maintained as subcutaneous transplants by inoculation of 1 mm.^{3} portions of actively growing tumour cortex into the right flank of recipient mice by means of a trocar

and cannula. Sarcoma 37 and the spindle-celled sarcoma BP1 were maintained in male mice, and adenocarcinoma MV212 in females.

Establishment of Serially Irradiated Tumour Lines

Four irradiated tumour lines were established. Sarcoma 37, BP1 and MV212 received doses at each stage approaching the tumour lethal values (" B ", " F " and " G " lines, respectively), and sarcoma 37 irradiated with a half-lethal dose at each stage (" D " line). Subcutaneous tumours were irradiated when the external measurements approximated 15–30 mm.^{2} in area (the product of the major and minor axes measured by calipers).

The doses administered at each stage in the irradiated series for the four tumour lines are given in Table I. 3000 r administered to sarcoma 37 produced tumour regression over a period of 15–20 days after treatment, usually to the point at which it ceased to become palpable. If growth resumed the tumour again became palpable within a few days. 2000 r administered to this tumour resulted in a slight tumour regression followed by growth cessation for a period of 12–20 days. The homologous tumours BP1 and MV212 reacted to sublethal doses in a less uniform manner; a cessation of growth, usually over a prolonged period, or greatly reduced growth rate followed a small initial regression. Tumours which became unpalpable did not recur. A proportion of tumours irradiated with doses well below the level required for all tumours to regress exhibited complete regression. For this reason the doses administered at each stage to these two homologous tumours were progressively reduced.

One tumour was selected at each stage, after recovery from irradiation, for transplantation into recipient mice by subcutaneous inoculation. Tumours developing in these mice were then irradiated and the series continued.

TABLE I.—*Irradiation Doses Administered to Each Stage of the Four Serially Irradiated Tumour Lines*

Sarcoma 37		Sarcoma BP1	Adenocarcinoma MV212
" B " line	" D " line	" F " line	" G " line
B 0 3000r	D 0 2000r	F 0 3000r	G 0 2000r
B 1 ,,	D 1 ,,	F 1 2500r	G 1 ,,
B 2 ,,	D 2 ,,	F 2 2000r	G 2 1500r
B 3 ,,	D 3 ,,	F 3 1750r	G 3 1250r
B 4 ,,	D 4 ,,	F 4 1500r	G 4 ,,
B 5 ,,	D 5 ,,	F 5 ,,	G 5 ,,
B 6 ,,	D 6 ,,	F 6 1250r	G 6 1500r
B 7 ,,	D 7 ,,	F 7 (from F 6	G 7 ,,
B 8 ,,	D 8 ,,	+ 1250r)	G 8 ,,
B 9 ,,	D 9 ,,		G 9 ,,
B 10 ,,	D 10 (from D 9		G 10 (from G 9
B 11 ,,	+ 2000r)		+ 1500r)
B 12 ,,			
B 13 ,,			
B 14 ,,			
B 15 ,,			
B 16 2500r			
B 17 (from B 16			
+ 2500r)			

Two sources of X-irradiation were used. For the establishment of the serially irradiated tumour lines a Siemens Stabilivolt Constant Potential Unit with a

Siemens EW 200 water-cooled glass tube were used. X-irradiation was generated at 190 kV and 8 mA. Filters were not employed and the dose rate in air at 25·5 cm. from the target was 240 r/min. having a quality of 0·25 mm. Cu H.V.L. Owing to a failure in this apparatus the radiosensitivity experiments and the irradiation of the last stages of the irradiated lines were conducted with X-ray apparatus employing a Siemens Doglas 200 kV tube maintained at the British Empire Cancer Campaign Research Unit in Radiobiology, at the Mount Vernon Hospital. Unfiltered radiation generated at 190 kV and 8 mA was again employed with this apparatus. The dose rate in air at 25·5 cm. from the target was 245 r/min. having a quality of 0·33 mm. Cu H.V.L.

Tumours were irradiated *in situ*, the mice being strapped to cork boards in the prone position ventral side down. The flap of the skin to which the tumour was adhering was lightly pulled away from the body and secured by adhesive plaster. This procedure was adopted to minimise the effect of scattered irradiation on the body. A lead shield with a suitable aperture over the tumour, protected the mouse.

External examination of the blood vessels in the skin flap, by means of a binocular microscope, showed that the degree of tension applied did not restrict the flow of blood.

Radiosensitivity evaluations

To assess the radiosensitivity of control and irradiated tumour lines, the proportion of " takes " of transplanted irradiated tumour fragments was taken as a criterion. A range of doses was employed up to the value where no tumours arose from the transplantation of the irradiated fragments; this will be referred to hereafter as the lethal level.

Thin slices, not more than 1 mm. thick, from control and irradiated line tumours were placed on a No. 5 filter paper in a sterile 2-in. petri dish. The paper was marked to avoid confusion of the tumour slices. A medium of 60 per cent Earle's saline (Earle, 1943) 40 per cent horse serum was added, sufficient to cover the tumour slices. Each dish contained collateral control and treated tumour fragments and separate dishes were employed for each dose level. The dishes were irradiated at room temperature at a distance of 22 cm. from the target at 190 kV and 8 mA without filters; the dose rate in air was 342 r/min. Subsequently the slices were cut into 1 mm. cubes and inoculated subcutaneously into the right flanks of 12 week old mice. Twelve mice were inoculated in control and treated groups for each dose level. These mice were kept for a period of two months after irradiation; after this period animals showing no apparent external tumour were killed and the inoculation site examined. No apparently negative mice showed evidence of tumour establishment at autopsy.

In order to examine the possibility of the tumour bed modifying the effects of irradiation on control and treated tumours, *in vivo* irradiations with subsequent transplantation were carried out on sarcoma 37 and its serially irradiated " B " line. In these experiments four matched tumours from each group, from 50–70 sq. mm. in area, were irradiated at each dose level by the method described for the establishment of the irradiated lines. Immediately after irradiation the tumour cortex was excised to a depth of about 2 mm. from the region which faced the X-ray tube. This material was pooled within each group and placed in a glass

homogeniser with 4 ml. of Earle's saline. Pulping was carried out until no perceptible fragments remained. This treatment had been found to give suspensions of single cells or clumps of not more than four cells. 0·1 ml. of the suspension was inoculated subcutaneously into the right flank of 30 mice in each group. These mice were examined over a period of two months for local tumour growth.

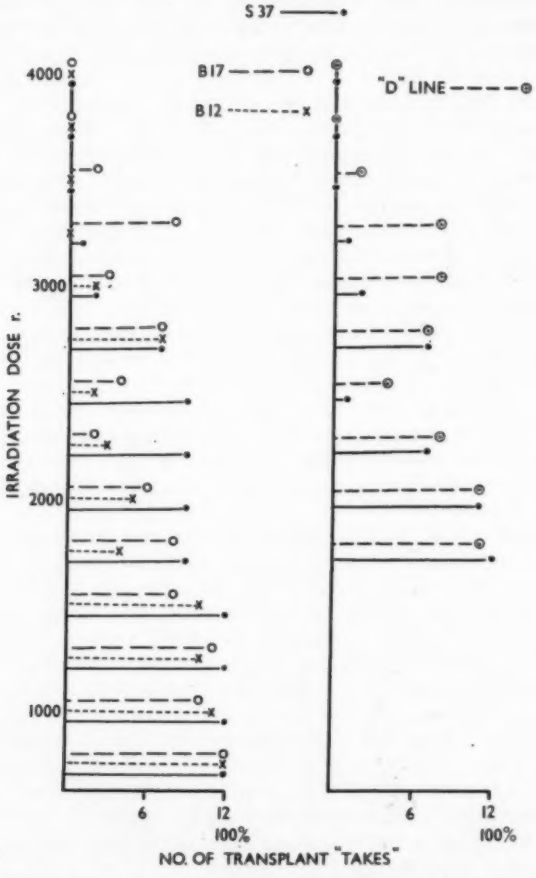

FIG. 1.—Radiosensitivity *in vitro* of sarcoma 37 and its serially irradiated lines " B " (sub-lethal dose at each stage) and " D " (half-lethal dose at each stage).

In vitro and *in vivo* radiosensitivity tests were conducted on sarcoma 37 and tumours from the 12th and 17th irradiation stages of the " B " line and only *in vitro* tests on the 10th stage of the " D " line. *In vitro* tests only were carried out on sarcoma BP1, the 7th irradiation stage of its " F " line, adenocarcinoma MV212 and the 10th stage of its irradiated " G " line. Owing to the restricted

availability of the irradiation source and supply of mice, tumours from untreated sub-line passages from the specified irradiation stages had to be used for these tests.

RESULTS

Fig. 1 illustrates the results from *in vitro* radiosensitivity tests on sarcoma 37, its derived " B " line tumours at the 12th and 17th irradiation stages (B 12 and B 17) and the 10th irradiation stage of the " D " line (D 10).

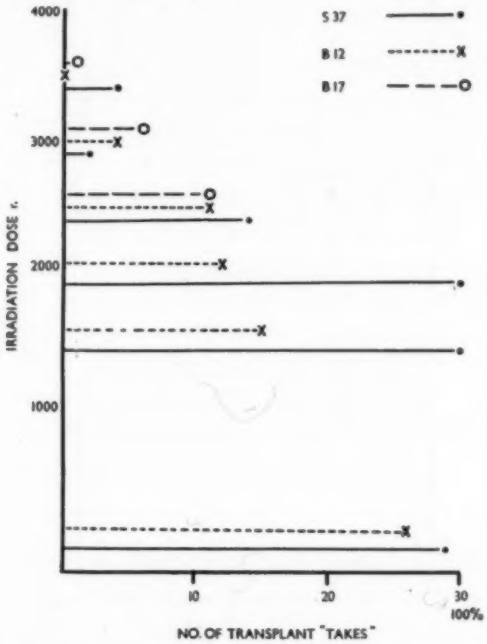

FIG. 2.—Radiosensitivity *in vivo* of sarcoma 37 and its serially irradiated " B " line.

B 12 tumours appeared to be more radiosensitive than the controls up to 2750 r. The lethal dose however was of the same order—3250 r (only one control tumour recurred at this dose level). B 17 tumours followed the same general pattern until the lethal dose was approached ; an apparent increase in radioresistance at 3250 r and 3500 r may be significant. Comparable results were obtained in the *in vivo* irradiation tests (Fig. 2) and B 12 tumours again exhibited an increase in radiosensitivity over the intermediate dose range of 1500–2500 r.

Increased radioresistance in " D " line tumours was observed over the intermediate dose range (Fig. 1) but again the lethal level was comparable with the controls.

The results of *in vitro* radiosensitivity tests on the two homologous tumours and their irradiated lines were comparable (Fig. 3 and 4). An increase in radio-

resistance was apparent in both irradiated lines with intermediate doses. At dose levels approaching the lethal values the response in control and treated groups was similar; the lethal dose was the same for sarcoma BP1 and its irradiated "F" line and varied by only 250 r between adenocarcinoma MV212 and its irradiated "G" line.

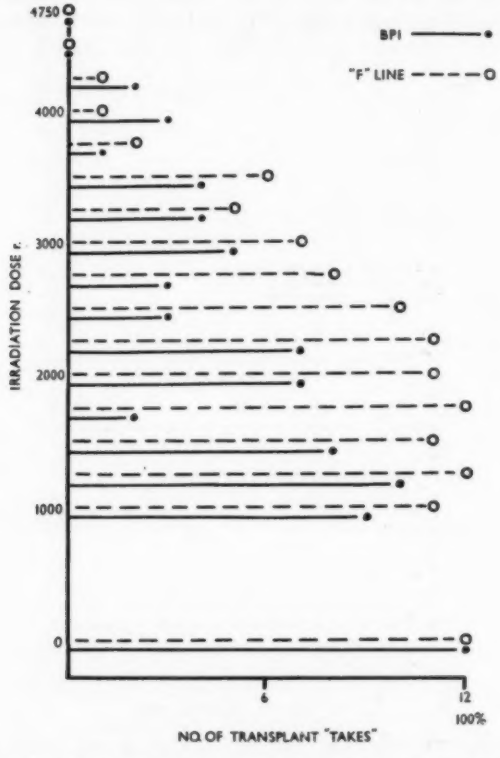

FIG. 3.—Radiosensitivity *in vitro* of sarcoma BP1 and its serially irradiated "F" line.

DISCUSSION

Two features emerge from these experimental findings. Firstly, the lethal dose levels of the irradiated tumour lines were not markedly different from their respective controls. Secondly, apparent differences in radiosensitivity were restricted to intermediate doses. Since the clinical observation of acquired radioresistance is related to complete tumour destruction, experimental comparisons should be made at this level. It would appear therefore from these experiments that the hypothesis of a selection by previous irradiations of more radioresistant cells, or a mutagenic effect, does not necessarily hold true. If the clinical evaluation of acquired radioresistance is based on experience of curability, with tumours

of comparable position and histological pattern, a degree of uniformity in radiosensitivity would be presupposed. However, many authors have described wide variations in radiosensitivity among tumours of similar histological pattern, both in the clinical and experimental fields (Smithers, 1946 ; Cramer, 1934 ; Tansley and Wilson, 1947 ; Cohen and Cohen, 1955). Cathie (1939) from clinical experience did not regard histology as a good guide to sensitivity and a similar observation was made by Goldfeder (1947) on experimental material.

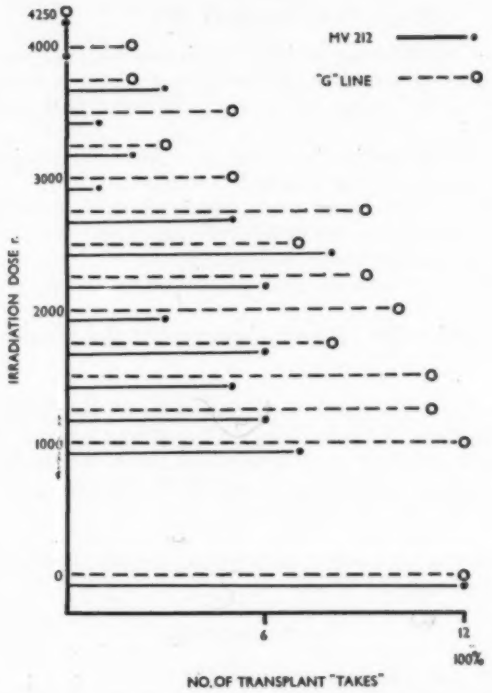

FIG. 4.—Radiosensitivity *in vitro* of adenocarcinoma MV212 and its serially irradiated "G" line.

The radiosensitivity comparisons between the control and irradiated lines indicate that a difference in response in the region of a tumour LD_{50} dose cannot be extrapolated to provide information in the region of the lethal dose. The apparent increase in radioresistance in the irradiated lines of the two homologous tumours was observed only with intermediate doses. This effect may have been due to a synergistic effect of an independent factor, acting against the establishment of the tumour cells in the recipient host. The intensity of this factor may have been low, so that its action might be masked at the higher irradiation doses. This hypothetical factor may have been an immunity response by the host to the

tumour implant. The previous irradiations might have modified the tumour cells with respect to their ability to produce antigens, or their reaction to the host antibodies. Cohen and Cohen (1953, 1954 *a*, *b* and *c*) have studied the relation between the host immunity response to a transplantable tumour and its radiosensitivity ; they considered that sensitivity was a function of the host resistance. If acquired radioresistance in clinical practice is a veridical phenomenon, it is suggested that immunological considerations may be involved in these isologous instances.

Summary

1. A method of establishing serially irradiated tumour lines of sarcoma 37 and two homologous tumours in an inbred mouse strain is described.

2. Radiosensitivity comparisons between the irradiated and control tumour lines have been made.

3. No marked differences in the lethal dose were observed between the four irradiated tumour lines and their respective controls.

4. Radiosensitivity differences were observed at intermediate dose levels. This was regularly consistent in the irradiated lines of the two homologous tumours and took the form of an increase in radioresistance.

5. The experimental results are discussed in relation to the problem of acquired radioresistance.

6. A possible relationship between these results and immunological factors is suggested.

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REFERENCES

Bagg, H. J.—(1938) *Amer. J. Roentgenol.*, **40**, 418.
Baillif, R. N.—(1958) *Abstr. Proc. Amer. Ass. Cancer Res.*, **2**, 277,
Cade, S.—(1948) ' Malignant Disease and its Treatment by Radium,' 2nd Ed., London (John Wright & Sons). Vol. 1, p. 153.
Cathie, A. B.—(1939) *Radiology*, **32**, 425.
Cohen, A. and Cohen, L.—(1953) *Brit. J. Cancer*, **7**, 452.—(1954*a*) *Ibid.*, **8**, 303.—(1954*b*) *Ibid.*, **8**, 313.—(1954*c*) *Ibid.*, **8**, 522.—(1955) *Ibid.*, **9**, 600.
Conger, A. D. and Luippold, H. J.—(1957) *Cancer Res.*, **17**, 897.
Cramer, W.—(1934) *Sci. Rep. Cancer Res. Fd., Lond.*, **11**, 127.
Dittrich, W., Hohne, G. and Schubert, G.—(1956) ' Progress in Radiobiology,' Edinburgh (Oliver and Boyd) p. 381.
Earle, W. R.—(1943) *J. nat. Cancer Inst.*, **4**, 165.
Goldfeder, A.—(1947) *Radiology*, **49**, 724.
Hill, E., Morton, J. J. and Witherbee, W. D.—(1919) *J. exp. Med.*, **29**, 89.
Lettré, H.—(1956) *Ann. N.Y. Acad. Sci.*, **63**, 1022.
Montgomery, F. O'B. and Warren, S.—(1953) *Radiology*, **60**, 421.

MOTTRAM, J. C.—(1932) *Brit. J. Radiol.*, **5**, 768.
RICE, C. M.—(1957) *Amer. J. Roentgenol.*, **78**, 831.
PATERSON, R.—(1949) 'The Treatment of Malignant Disease by Radium and X-Rays,' London (Edward Arnold & Co.), p. 14.
RUSS, S.—(1924) *Brit. J. Radiol.*, **29**, 275.
SMITHERS, D. W.—(1946) 'The X-Ray Treatment of Accessible Cancer," London (Edward Arnold & Co.), p. 30.
SNELLMAN, B.—(1935) *Acta Radiol., Stockh.*, **16**, 545.
TANSLEY, K. AND WILSON, C. W.—(1947) *Radiology*, **49**, 62.
WINDEYER, B. W.—(1954) *Acta Radiol., Stockh.*, Suppl. 116, 108.

SPONTANEOUS TUMOURIGENESIS IN AGED GUINEA-PIGS

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SUBTOTAL castration is followed in various species by an ovarian-hypophyseal hormonal imbalance as evidenced by the appearance of luteic cysts (cat: Lipschutz and Voss, 1925; guinea-pigs: Lipschutz, 1931, Osnowikoff, 1934; rat: not published). Other signs of an ovarian-hypophyseal imbalance also may be present in subtotal castration as haemorrhagic follicles (guinea-pig: Bruzzone and Lipschutz, 1954; mice Balb A: Lipschutz, 1958) and even luteomata (mice Balb A). An increased ovarian blood supply not dependent on the hypophysis also might be implicated as a causative factor in ovarian change (Nelson, 1930; Haterius, 1930). But the haemorrhagic follicles and luteic cysts which appear in the ovarian remnant give sufficient evidence that under the given experimental conditions an ovarian-hypophyseal imbalance is in play.

Cystic glandular hyperplasia of the endometrium has been found in guinea-pigs several months after the ovarian operation (Burch, Wolfe and Cunningham, 1932; Lipschutz and Osnowikoff, 1932). Adenomatous polyps, penetration of proliferated uterine glands into the myometrium, and—in one case—epithelioma of the cervical mucosa also were found (Lipschutz, 1937, 1938). Various other tumours in operated guinea-pigs have been subsequently described by the authorities (Morató, 1941; Nadel, 1949; Ponse and Dovaz, 1951; Bruzzone and Lipschutz, 1954; see also Lipschutz, 1950, 1957, 1958).

The most spectacular result was uterine adenocarcinoma as found in animals necropsied as late as three to almost four years after operation, when the animals were already 4 to 5 years old. Thus we had to ask ourselves whether similar tumoural growth may occur also spontaneously in non-operated aged guinea-pigs (Bruzzone and Lipschutz, 1954), or how far tumoural growth in operated animals necropsied when already very old might have been dependent on age. Indeed, appearance of tumoural growth in aged non-operated guinea-pigs would not invalidate our assumption that an ovarian-hypophyseal imbalance may cause uterine and extra-uterine tumourigenesis. Spontaneous tumours are very rare in the guinea-pig. Papanicolaou and Olcott (1940, 1942) have reported extra-uterine leiomyomata attached to the stomach or to the intestine in seven females 4 to 7 years old. There was also adenoma of the suprarenal. The total number of spontaneous tumours in guinea-pigs in the extensive work of Papanicolaou was of 100 among 7000 animals. For other findings of spontaneous tumours in guinea-pigs we may refer to Warren and Gates (1941), Olcott and Papanicolaou (1943), Riesco and Schwarz (1944) and Rogers (1951, 1952).

Spontaneous uterine growths have been noticed in the guinea-pig in this laboratory (Lipschutz, 1941); there was a small pedunculated uterine leiomyoma in one case, and intramural nodules of hyperplastic muscle fibres in another

ase. No other finding of this kind has come to our knowledge. Neither has uterine denocarcinoma as found in females with subtotal castration (Bruzzone and ipschutz, 1954) been so far described in non-operated animals.

Thus, at the first sight the tumoural spectrum in normal aged guinea-pigs eems to be quite different from that in operated animals of ours or of the authorities. However, all work both of ours and of the authorities has been performed on non-elected breeds. For this reason it seemed necessary to reinvestigate the whole uestion. This reinvestigation has been done in two different ways.

First (Lipschutz, 1958): Subtotal castration was performed in a selected breed of mice (Balb A). Work is greatly facilitated by the short life cycle in mice which is of 2 years or rather less. The animals were necropsied 17 months after operation when reaching the advanced age of 19 to 20 months. There were haemorrhagic follicles in 17 out of 28 animals. In 16 animals luteomata were produced; 3 of these luteomata were "exclusive" ones, i.e. no follicles or corporal lutea were present. Abnormal uterine structures (polyps, glandular invasion into the myometrium, stratification and cornification of the endometrial glands, large leiomyoma) were found in no less than 14 out of the 28 operated animals. In a group of 25 non-operated animals 17 to 24 months old there was no animal with luteoma; an haemorrhagic follicle and a luteic cyst were present in only 2 out of the 25 non-operated animals. The beginning of an hormonal imbalance was evidenced by a slight degree of cystic glandular hyperplasia occurring in most of these aged animals; but pseudostratification of the endometrium was found in only 2 animals and adenomatous growth beneath the endometrium in 1 animal. The difference between the group of operated and non-operated aged animals was overwhelming. The significance of the abnormal findings in the non-operated group shall be discussed at the end of this paper.

Second: Non-operated guinea-pigs were kept in the Institute to be necropsied at the age of 4 to 6 years. The results obtained with these 25 aged animals are given in the present paper.

General survey

There were in our group of aged guinea-pigs 14 animals about 4 years old, and 9 animals about 5 or 6 years old (Table I). The majority were born in this Institute, but the exact date of birth was not known. Some were separated when about 1 year old; their weight was then 500 g. Others, also born in the Institute, were separated when about 2 years old, as also several animals bought from a dealer; the weight of these animals was then of about 700 g. to 800 g. The "initial" weight in the table is indeed not exact as not all the animals were weighed immediately after being separated to form the group of aged animals. During the three to four years for which the animals were kept in the laboratory after separation there was the general tendency to lose weight.

In the group of 14 animals necropsied at the age of 4 years there was only 1 animal with a uterine growth visible to the naked eye (Table I; Table II, animal VII), whereas at 5 to 6 years uterine growths were seen at necropsy in 3 out of 9 animals (Table I; Table II, animals VIII, IX, X).

Notable was also the fact that in almost all the 25 aged animals there were ovarian cysts visible to the naked eye, sometimes of a very large size (see section "The ovary").

TABLE I.—*General Survey of Aged Non-operated Animals*

	Age group (years)	Weight			Number of animals			Localization of extra-uterine growths
		Initial (g.)	Maximum (g.)	Final (g.)	Total	With unterine growths visible by naked eye	With extra-uterine growths	
I	4	ab. 550	630 to 1030	390 to 965	14*	1	2	1 stomach, 1 lung
II	5–6	ab. 800	940 to 1300	695 to 1090	9	3	2	1 intestine, 1 thyroid
III	?	—	—	715 and 970	2	1	1	1 suprarenal and ovary

* Two of these animals only 3½ years old.

TABLE II.—*Uterine Growths in Aged Non-operated Guinea-pigs*

Series	Designation of animal	Age (years)	Type of growth	Details
CCXLI.3	I	4	Endometriosis	—
CCXLI.7	II	4	,,	—
CCXLI.8	III	4	,,	—
CCXLI.11	IV	4	,,	(Fig. 6)
CCXLI.4	V	4	Adenomatous polyp	(Fig. 8)
CCXLI.13	VI	4	Ditto	*Possibly beginning of dedifferentiation*
CCXLI.6	VII	3½	*Adenocarcinoma*	(Fig. 3)
CCXLI.16	VIII	5	Ditto	(Fig 4)
CCXLI.16	VIII	5	Leiomyoma	Between horn and cervix
CCXXXI.5	IX	5	Endometriosis	—
CCXXXI.5	IX	5	Leiomyoma	Cervix; of enormous size (Fig. 1)
CCXLI.18	X	6	Endometriosis Adenomyoma	*Possibly some dedifferentiated glands* (?) (Fig. 7)
CCXLI.17	XI	6	Adenomatous polyp	With *nodules of dedifferentiated glands* (?) (Fig. 5)
CCXLI.20	XII	6	Ditto	—

Uterine Growths

Leiomyoma.—There were 2 animals with leiomyoma, both of them 5 years old (Table I; Table II, animals VIII and IX). One of these tumours was exceedingly large (IX; Fig. 1). The tumour filled and greatly distended the uterine cervix which was reduced to a large thin-walled sac. The tumour was easily enucleated; its weight was of 270 g., i.e. exactly 25 per cent of the weight of the animal. Microscopically it was a massive leiomyoma; at different places there was loose and somewhat oedematous fibrous tissue rich in blood vessels.

The senior writer (A. L.) out of his experience of almost forty-five years with guinea-pigs remembers having seen only one other uterine tumour of similar size which at necropsy was classified as a myoma, or fibromyoma (work of Riesco).

A tiny spontaneous uterine leiomyoma attached to the parametrium has been mentioned above (Lipschutz, 1941). A leiomyoma of minor size has been described in a guinea-pig with subtotal castration by Morató (1941). Three other cases belonging to an unpublished series of ours may be added (Table III). These leiomyomata were found in animals necropsied 36, 42 and 45 months after subtotal castration. Localization of these tumours (Fig. 2) recapitulates that in animal IX of the present series (Fig. 1). In one of these leiomyomata there was a pronounced partial necrobiosis and necrosis (Table III, animal I).

TABLE III.—*Leiomyomata in an Unpublished Series of* 18 *Animals Aged* 3 *to* 4 *Years with Subtotal Castration, Operated when* 2 *Months Old*

Series	Designation of animal	Age (years)	Site	Details
CCXXX.7	I	$3\frac{8}{12}$	Cervix	Partial necrosis and ossification in the centre
CCXXX.12	II	$3\frac{8}{12}$	Cervix	(Fig. 2)
CCXXX.14	III	$3\frac{1}{12}$	Two tumours: 1. Between uterine horn and cervix 2. Vagina	There was also a tumour of Langerhans islets with ossification (Fig. 13)

The second leiomyoma of the present series of non-operated animals was intramural, belonging to the distal part of the uterine horn and descending into the cervical wall (animal VIII of Table II). In the upper part there was no limit between the tumour and the myometrium, in such a way that uterine glands were englobed by leiomyomatous masses.

Adenocarcinoma.—In our former work with subtotal castration there were two cases with large dedifferentiated adenocarcinomata of the uterine horn, another case with a smaller nodule, and a fourth case which we classified as an "initial" stage of adenocarcinoma (see Table III in Bruzzone and Lipschutz, 1954). In the present work with non-operated animals not a single animal with large uterine adenocarcinoma was found at necropsy. But since we were now very carefully searching for neoplastic uterine growth it was decided to select for histological examination all those parts of uterine horns which seemed to be somewhat distended even when in a very inconspicuous manner. Thanks to this search 2 cases with adenocarcinoma were found. One of these animals (Table II; animal VII) was only $3\frac{1}{2}$ years (Fig. 3) and the other 5 years old (VIII; Fig. 4). These nodules are structurally identical with those in our former work with operated animals of $4\frac{1}{2}$ and 6 years but without reaching the large size as in the latter.

The "initial" stage of adenocarcinoma, as formerly described in an operated animal 4 years old, was present in 2 non-operated animals of the present series (Table II; animals X and XI); these aninals were 6 years old. The picture as present in one of these animals (XI) is of considerable interest. Fig. 5 shows two types of glands: nodules of proliferated glands whose cells are rich in protoplasm are intermingled with nodules in which the glandular cells are seemingly on the way to dedifferentiation. The latter are those immediately beneath the endometrium as if prepared to become the endometrial or superficial adenocarcinomatous nodule.

Endometriosis.—Invasion of glands into the myometrium was found in 4 animals 4 years old (I to IV ; Fig. 6), and in 2 animals 5 and 6 years old (Table II ; animals IX and X). In one of these cases (X) the picture was that of adenomyoma but possibly with dedifferentiation (Fig. 7).

Adenomatous polyps.—They were found in 4 animals (Table II ; animals V and VI, and XI and XII ; animal V : Fig. 8). As already explained in the section on adenocarcinoma such an adenomatous polyp may consist both of nodules of normal and dedifferentiated glands (animal XI, Fig. 5 ; and possibly also animal VI). In another case, of unknown age and not mentioned in Table II (belonging to group III of Table I) the septum of the uterine cervix descended deep into the latter reaching its distal entrance.

The ovary

The condition of the ovary is summarized in Table IV.

TABLE IV.—*Condition of the Ovary in Non-operated Aged Guinea-pigs.*

Age group (years)	Number of animals				With active vaginal mucosa
	Total	With cysts of rete	With corp. lut.		
			Total	Degenerate	
4	14	14	11	5	6
5–6	9	8	6	3	6
?	2	1	1	—	1

The most conspicuous deviation from the normal is, as already mentioned, the presence of cysts. They were in general bilateral (Fig. 9). The cysts were large in both age groups.

Both the site the cysts occupy in the ovary and their cubic epithelium speak in favour of their belonging to the rete ; they are certainly not of follicular origin.

Primary and secondary follicles and also Graafian follicles were always present. But their number was diminished when comparing with the ovary of fully grown younger animals. Graafian follicles in atresia also offered sometimes seemingly abnormal pictures. However, it would be difficult for us to give an exhaustive picture of the deviations from the normal picture of younger animals without a special comparative study. There was in any case the fact that corpora lutea were present in most of the animals, though in half the animals with corpora lutea the latter were in a state of degeneration.

The oestrogenic activity of the ovary was evidenced by the active condition of the vaginal mucosa : prooestrous, oestrous and metoestrous were present in no less than half the 25 animals.

There was in one case an ovarian tumour ; for fuller details see Table V and section " Extra-uterine Growths ".

Extra-uterine growths

Of the 6 extra-uterine growths (Table V) 1 was a lung tumour which does not offer any interest in our context. The remaining 5 tumours may be tentatively classified, together with uterine tumours, as due to a hormonal imbalance ; for this reason they deserve special attention when discussing the pertinen

problems of spontaneous tumourigenesis in aged animals. This may refer in the first place to tumours of the thyroid, suprarenal and ovary but also, though to a certain degree only, to the gastro-intestinal tumours, one of which was a leiomyoma of the stomach, the other a leiomyoma of the small intestine.

Leiomyomata of the stomach and the intestine in aged guinea-pigs have been masterly described by Papanicolaou and Olcott (1940, 1942). The leiomyomata found in our work (Table V) are identical in microscopical structure with those described by these authorities. But it may be mentioned that at the place where one of the tumours of the stomach (Fig. 10) was attached to the wall the latter had undergone considerable changes ; the mucosa became very thin though the continuity of the gastric wall had remained intact. It is remarkable that a similar condition but much more pronounced has prevailed also with the leiomyoma of the intestine : the mucosa above the tumour had been destroyed by the growth protruding into the intestinal cavity (Fig. 11). The intestinal cavity in the vicinity of the tumour was full of debris of the mucosa.

TABLE V.—*Extra-uterine Tumours in Non-operated Aged Guinea-pigs*

Series	Designation of animal	Age (years)	Site of tumour	Type of tumour	Other growths in the same animal
CCXLI.1	I	4	Lung	Bronchial adenoma	0
CCXLI.5	II	4	Stomach (Fig. 10)	Leiomyoma	0
CCXLI.20	III	6	Small intestine (Fig. 11)	,,	Uterine adenomatous polyp : see Table II, animal XII
CCXLI.19	IV	5	Thyroid, bilateral	Colloid Adenoma	0
CCXXXI.D	V	?	Right suprarenal Left ovary (Fig. 12)	Adenoma of cortex Papillary cystadenoma	0

The lobes of the thyroid of an animal 5 years old had a weight of 266 and 330 mg., i.e. many times that of the normal gland. It was a colloid adenoma, or very pronounced hyperplasia.

The tumour of the suprarenal cortex was an adenoma most probably of the fasciculata.

The ovarian tumour as present in the same animal was a papillary cystadenoma (Fig. 12).

In one of the operated animals only 3 years old a tumour of Langerhans islets was found (Table III, animal III ; Fig. 13).

DISCUSSION

Tables I, II and V give evidence that in aged normal guinea-pigs up to 4 years old uterine tumours appeared only exceptionally. There was not a single animal with uterine leiomyoma among 14 non-operated animals up to 4 years old (Table I) and there were 3 animals with similar leiomyomata among 18 operated animals in the fourth year (Table III). Indeed the groups are too small to allow of any definite conclusion as to the influence the experimentally induced ovarian-hypo-

physeal imbalance might have on the origin and evolution of uterine leiomyomata. More relevant is the fact that in non-operated animals about 5 to 6 years old atypical epithelial growth of the uterus, including superficial adenocarcinoma, was as frequent as in experimental animals which had undergone subtotal castration.

There was however, between operated and non-operated animals, a difference whose fundamental importance is discussed now : the two cases of adenocarcinoma as present in non-operated animals and mentioned in Table II (animals VII and VIII) were of so insignificant a size that they probably would have escaped our notice at necropsy should we not have met with similar tumours of large size in our previous work with subtotal castration (Bruzzone and Lipschutz, 1954) and should we not have taken beforehand the decision to search for any initial stage of adenocarcinoma.

Thus there is now, on the one hand, the definite fact that atypical growth of the uterine epithelia, including adenocarcinoma, may originate spontaneously

EXPLANATION OF PLATES

FIG. 1.—Leiomyoma of cervix of enormous size in non-operated animal, 5 years old (Table II, animal IX ; series CCXXXI.5). × ¼.

FIG. 2.—Leiomyoma of cervix in operated animal, 3 years old (Table III, animal II ; series CCXXX.12). × ¼.

FIG. 3.—Nodule of uterinea denocarcinoma in non-operated animal 3½ years old (Table II, animal VII ; series CCXLI.6). × 34. The nodule was structurally identical with that in Fig. 4.

FIG. 4.—Nodule of uterine adenocarcinoma in non-operated animal, 5 years old (Table II, animal VIII ; series CCXLI.16). × 96. Dedifferentiated glands ; below, endometrial glands of normal aspect.

FIG. 5.—Adenomatous polyp of uterus in non-operated animal, 6 years old (Table II, animal XI ; series CCXLI.17). —A. × 34. Two types of glands. —B. × 96. Top : endometrial glands of normal type ; below : glands possibly of abnormal type.

FIG. 6.—Endometriosis in non-operated animal, 4 years old (Table II, animal IV ; series CCXLI.11). —A. × 34. —B. × 100. To the left : endometrial glands in the submucosa ; to the right : endometrial glands between the circular and longitudinal layer of the myometrium.

FIG. 7.—Uterine adenomyoma in non-operated animal, 6 years old (Table II, animal X ; series CCXLI.18). —A. × 10. —B. × 100. Uterus with high cells of endometrium and normal glands. —C. × 100. Limit between normal glands in the submucosa, and glands of the adenomyoma undergoing change. —D. × 100. Glands seemingly abnormal, amidst the leiomyomatous tissue.

FIG. 8.—Adenomatous polyp in non-operated animal, 4 years old (Table II, animal V ; series CCXLI.4). × 10.

FIG. 9.—Non-operated animal, 4 years old (Table II, animal V ; series CCXLI.4). Both ovaries with large cysts. × 1. (See also Fig. 8.)

FIG. 10.—Non-operated animal, 4 years old (Table V, animal II ; series CCXLI.5). Dorsal view of stomach. Two leiomyomata. × ⅔.

FIG. 11.—Non-operated animal, 6 years old (Table V, animal III ; series CCXLI.20). Small intestine with large leiomyoma. —A. × 5. —B. × 35. Local destruction of intestinal mucosa.

FIG. 12.—Aged non-operated animal ; exact age unknown (Table V, animal V ; series CCXXXI.D). —A. Ovary with large cyst. × 4·8. —B. Tumour attached to the ovary, most probably cystadenoma. × 34.

FIG. 13.—Operated animal, 3 years old (Table III, animal III ; series CCXXX.14). Tumour of the islets of the pancreas. —A. Somewhat more than natural size. —B. × 96. Tumour on the bottom. —C. × 96. Ossification amidst the proliferated islet cells.

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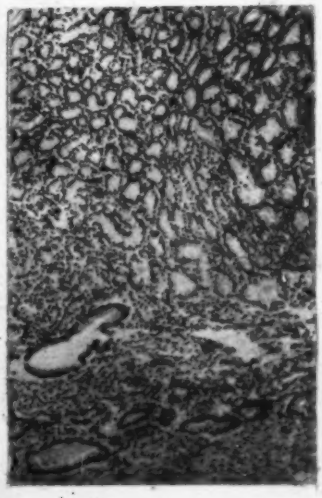

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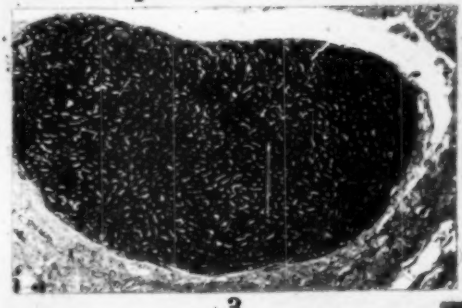

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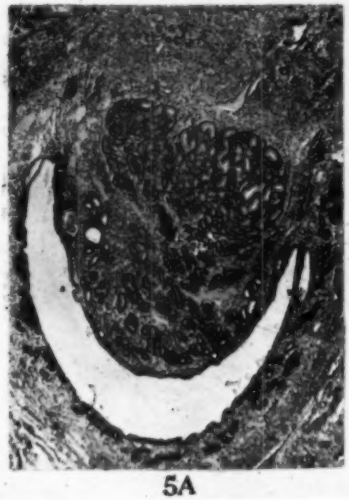

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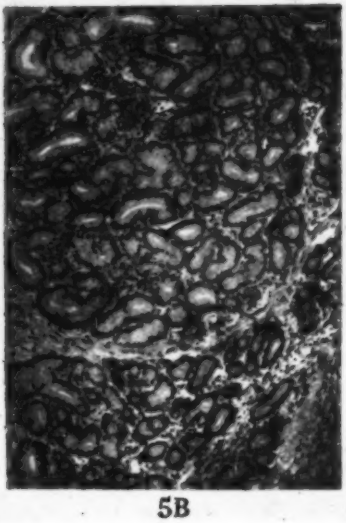

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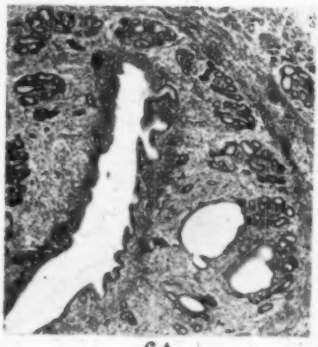

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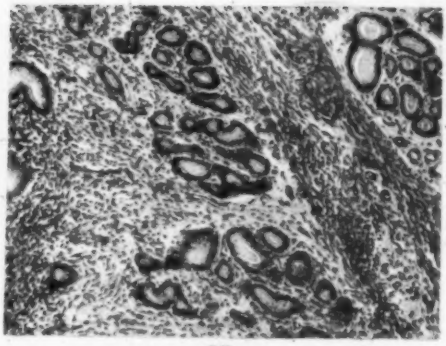

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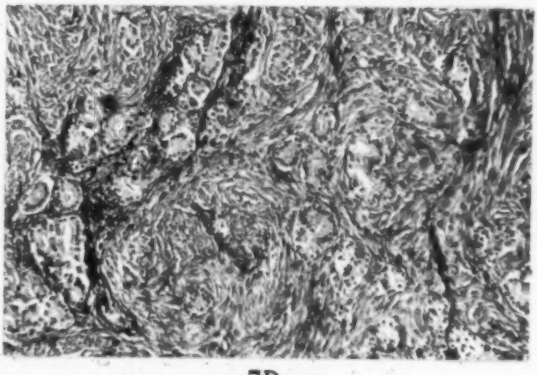

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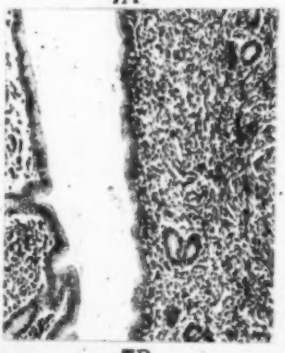

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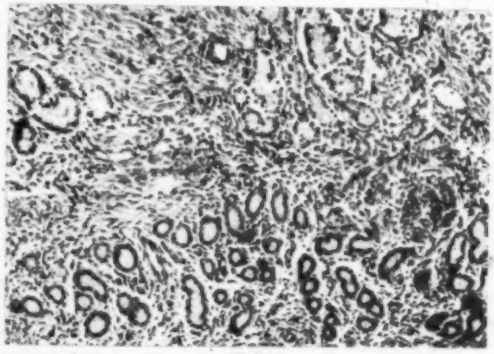

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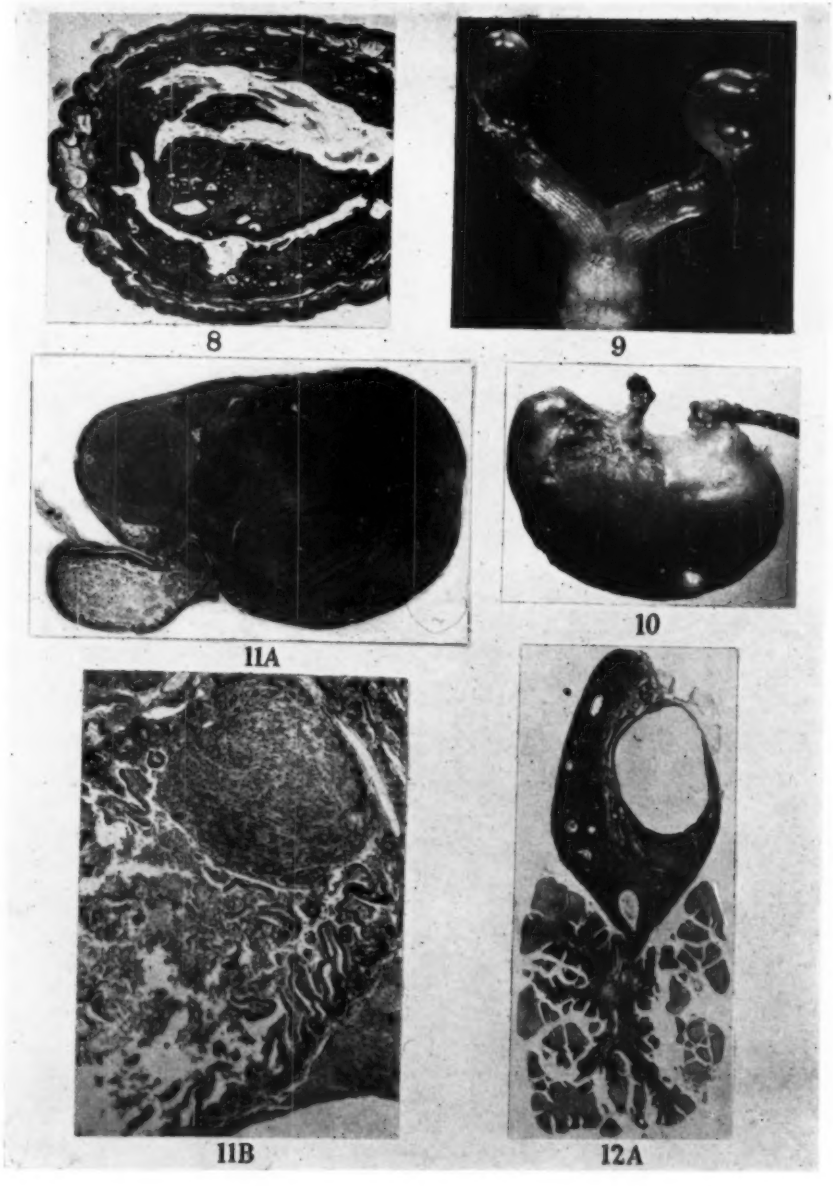

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Lipschutz, Iglesias, Rojas and Cerisola.

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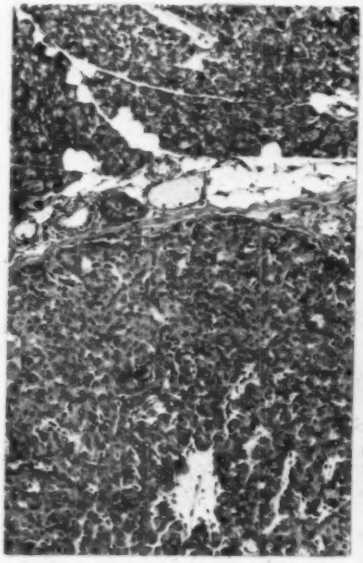

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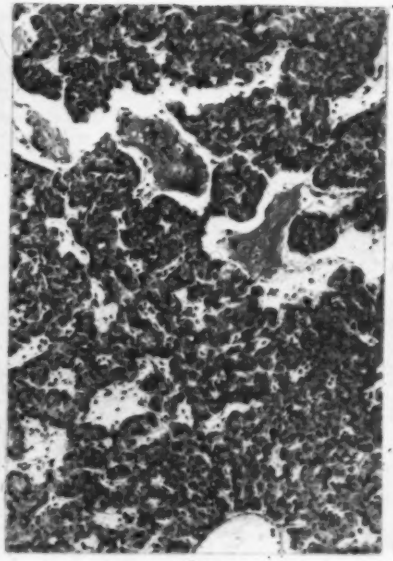

13C

n very old guinea-pigs. And on the other hand one may tentatively assume that typical and tumoural growth, as originating spontaneously in aged animals nd as probably due to a spontaneous ovarian-hypophyseal hormonal imbalance s accelerated and accentuated by subtotal castration by which an ovarian-ypophyseal imbalance is established in younger animals also, as fully evidenced y the condition of the ovary, in our former work. In favour of our conclusion here is also the difference in degree between uterine epithelial tumourigenesis n aged non-operated animals as described in the present series, and in aged perated animals as described in former papers.

Even more decisively in favour of this conclusion are our findings in mice Balb A with subtotal castration. At the end of their short life cycle the difference between the uterus in operated and non-operated animals is overwhelming (Lipschutz, 1958). In Balb A with subtotal castration 20 months old there was, as already explained, in no less than half of the animals atypical epithelial growth with uterine polyps, stratification of endometrium or glands, including cornification. This is, by itself, nowadays of trivial interest : it is but the sequel of those structural and functional transformations the ovarian remnant undergoes in the course of a year and a half after operation, i.e. in about three quarters of the life span of these animals. However, these findings are of utmost interest when compared with the condition of the uterus in non-operated aged animals of the same strain, in which occur only a slight degree of cystic glandular hyperplasia, sometimes pseudostratification of the endometrium and of the glands and very exceptionally adenomatous growth. In other words, in normal aged animals there is a uterine condition which one may interpret as the sequel of an ovarian-hypophyseal imbalance at its very beginning! The condition of the ovary in non-operated aged mice is in full agreement with the above conclusion.

As shown formerly by Loeb (1948) and especially as evidenced by the outstanding work of the Mühlbock group very conspicuous changes occur in the ovary of aged mice of various strains (Thung, Boot and Mühlbock, 1956 ; Thung, 1956, 1958 ; Mühlbock, 1957 ; see also Green, 1957). In our aged Balb A mice these changes are less pronounced than in those strains with which the Mühlbock group is working or than in our C57bl mice, though indeed the gerontological start is made also in aged Balb A mice. This is expecially evidenced by the appearance of clusters of clear cells in the stroma. There is certainly an enormous distance between the condition of the ovarian remnant in our operated Balb A mice, and the ovary of non-operated aged animals of the same or various other strains. But there can scarcely be any doubt about the fundamental fact that there is in aged animals of all the domesticated strains hitherto examined an ovarian-hypophyseal imbalance, though so far it would not be possible to establish the dynamics from which this imbalance originates (see important work of Jones and Krohn, 1959 ; and former work on different lines of Lipschutz, Kallas and Paez, 1929, with bibliography). But in any case it seems true that the ovary in the aged animal treads upon a perilous path which may lead to ovarian tumourigenesis (Lipschutz, 1958 ; Lipschutz, Rojas, Cerisola and Iglesias, 1958). And in so far as the ovarian growth, be it luteoma or granulosa-cell tumour, is functional the same path leads also to extra-ovarian tumourigenesis.

From the point of view of gerontology all these observational and experimental statements are of utmost interest. Knowledge acquired in different strains of mice strengthens the assumption that there might be a genetically determined

ovarian-hypophyseal hormonal imbalance leading to tumourigenesis, some mutagenic influences of domestication being in play; or that some ambiental influences are in play on which hypophyseal or ovarian function depends.

One cannot but feel inclined to make use of the comparative knowledge about operated and non-operated aged mice for explaining the results as obtained in our aged guinea-pigs. The ovary in all our aged non-operated guinea-pigs was different from the normal ovary of fully grown but younger guinea-pigs. There is first of all the cystic condition of the ovary. Cysts of the rete, and likewise extra-ovarian cysts of Wolffian origin have been described repeatedly in animals with an experimental hormonal imbalance and especially in intrasplenic ovarian grafts (Iglesias, Mardones, Bruzzone and Lipschutz, 1953). Similar ovarian cysts are present also in ovarian remnants after subtotal castration; extra-ovarian cysts also have been found under these experimental conditions (Ponse and Dovaz, 1951). There were in our aged animals also nodules seemingly due to proliferation of rete tubules as seen in intrasplenic grafts and in ovarian remnants after subtotal castration (Lipschutz, 1938, 1950; Iglesias, Mardones, Bruzzone and Lipschutz, 1953); these nodules were indeed less conspicuous in the present series of aged animals. Besides the ovarian cysts in the ovaries of aged animals the follicular development also offered notable deviations from the picture of the normal ovary in fully grown females: the number of primary and secondary follicles was diminished, Graafian follicles were sometimes of an abnormal aspect. Certainly among our non-operated aged guinea-pigs there was not a single animal whose ovary would have given the same clear cut structural evidence of an ovarian-hypophyseal hormonal imbalance as do ovarian remnants with haemorrhagic follicles or luteic cysts. However, the condition of the uterus as summarized in our Table II speaks decidedly in favour of an imbalanced hormonal functioning of the ovary; and one scarcely exaggerates when assuming that there was in our aged non-operated guinea-pigs an ovarian-hypophyseal imbalance comparable to that which is established in guinea-pigs wih subtotal castration. The occasional presence of tumoural growth of the thyroid and of the suprarenal in aged non-operated guinea-pigs (Table V) also may be taken as proof of such an assumption. There is now sufficient knowledge about the normal functioning of an hypophyseal organotrophic complex being dependent on the hormonal elements of another organotrophic complex; there are the tumours of the suprarenal cortex induced by castration as known from classical work of Woolley (1950) in mice; there is the partial redress of the hormonal imbalance of the gonadotrophic hypophyseal function by the administration of a corticoid in mice with intrasplenic ovarian grafts (Mardones and Lipschutz, 1956).

When discussing the dynamics of tumourigenesis in guinea-pigs with subtotal castration especially of advanced age, we must now be aware that we are dealing with a complex phenomenon: there is the ovarian hypophyseal hormonal imbalance established by the operation as evidenced by the condition of the ovarian remnant; but there is likewise the hormonal imbalance supposedly present also in the non-operated animal. With operated guinea-pigs we do not know how far the experimental factor and how far the age factor are in play in each one of neoplastic events. However, with operated mice Balb A there is full evidence that the atypical uterine growth paralleled with the luteomatous condition of the ovarian remnant is prominently, though again not entirely, due to the experimentally established hormonal imbalance.

Very outstanding work done by South-African workers on various strains of rats must be mentioned here. Their findings favour greatly the assumption that both age and environmental conditions, especially dietary ones, may be responsible for tumourigenesis (Gilbert, Gilman, Lustalot and Lutz, 1958).

The relevance of all these observations in aged guinea-pigs, mice and rats for a concept of the dynamics of ovarian and uterine tumourigenesis in the aged woman is fully evident.

SUMMARY

Tiny nodules of uterine adenocarcinoma may originate spontaneously in non-operated guinea-pigs 4 to 6 years old.

These neoplastic nodules can be discovered only when making a systematic search both at necropsy and at microscopical examination.

Since these nodules are structurally coincident with large adenocarcinoma as formerly found in guinea-pigs with subtotal castration the tentative assumption is made that there is in aged guinea-pigs a tumourigenic ovarian-hypophyseal imbalance which is genetically determined in the course of domestication, or which is due to ambiental influences.

This assumption is strengthened by the following facts: (1) in non-operated aged guinea-pigs other tumours also occurred which are known to be dependent on the hormonal homeostasis in the body as uterine and extra-uterine leiomyoma, thyroid adenoma, suprarenal adenoma; (2) there were in these animals also endometriosis and adenomatous polyps which are known to be dependent on an ovarian-hypophyseal imbalance; (3) there was in all aged guinea-pigs a very pronounced cystic condition of the ovary as found in animals with an experimentally induced ovarian-hypophyseal imbalance.

The assumption that there is in advanced age an ovarian-hypophyseal imbalance paralleled with tumourigenesis is strengthened also by former comparative observations on mice with subtotal castration and on non-operated aged mice.

Thanks are due to Dr. Elvira Mardones, now of the Medical School of Universidad de Chile, who was in charge of the selection of animals for the old age groups among guinea-pigs and likewise of operations and necropsies in mice. Thanks are due also to the technical staff of the Institute for indefatigable help at necropsies, for histological, photographical and secretarial work.

REFERENCES

BRUZZONE, S., AND LIPSCHUTZ, A.—(1954) *Brit. J. Cancer*, **8**, 613.

BURCH, J. C., WOLFE, M. M., AND CUNNINGHAM, R. G.—(1932) *Endocrinology*, **16**, 541.

GILBERT, C., GILLMAN, J., LOUSTALOT, P. AND LUTZ, W.—(1958) *Brit. J. Cancer*, **12**, 565.

GREEN, J. A.—(1957) *Anat. Rec.*: **129**, 333.

HATERIUS, H. O.—(1930) *Ibid.*, **47**, 318.

IGLESIAS, R., MARDONES, E., BRUZZONE, S., AND LIPSCHUTZ, A.—(1953) *Arch. Anat. micr. Morph. exp.*, **42**, 3.

JONES, E. C. AND KROHN, P. L.—(1959) *Nature*, **183**, 1155.

LIPSCHUTZ, A.—(1931) *Endokrinologie*, **9**, 258.—(1937) *Gynéc. et Obstét.*, **36**, 407, 481.—(1938) *Ibid.*, **37**, 17.—(1941) *Arch. Path.*, **31**, 702.—(1950) 'Steroid Hormones and Tumors'. Baltimore (Williams & Wilkins).—(1957) 'Steroid Homeostasis, Hypophysis and Tumorigenesis'. Cambridge (Heffer).—(1958) *Acta Un. int. Cancr.* (in press).

Idem, KALLAS, H., AND R. PAEZ.—(1929) *Pflügers Arch. ges. Physiol.*, **221**, 695.

Idem AND OSNOWIKOFF, B.—(1932) *C. R. Soc. Biol., Paris*, **111**, 350.

Idem, ROJAS, G., CERISOLA, H. AND IGLESIAS, R.—(1958) *Acta Un. int. Cancr.* (in press).

Idem AND VOSS, H. E.—(1925) *Brit. J. exp. Biol.*, **3**, 35.

LOEB, L.—(1948) *Arch. Path.*, **46**, 401.

MARDONES, E., AND LIPSCHUTZ, A.—(1956) *Brit. J. Cancer*, **10**, 517.

MORATÓ, J.—(1941) *Endocrinology*, **29**, 6191.

MÜHLBOCK, O.—(1957) *Ciba Found. Coll. on Ageing*, **3**, 115.

NADEL, E. M.—(1949) *J. nat. Cancer Inst.*, **9**, 271.

NELSON, W. O.—(1930) *Anat. Rec.*, **45**, 272.

OLCOTT, C. T., AND PAPANICOLAOU, G. N.—(1943) *Cancer Res.*, **3**, 321.

OSNOWIKOFF, B.—(1934) *An. Med. interna*, **3**, 735.

PAPANICOLAOU, G. N., AND OLCOTT, C. T.—(1940) *Amer. J. Cancer*, **40**, 310.—(1942) *Arch. Path.*, **34**, 218.

PONSE, K., AND DOVAZ, R.—(1951) *Ann. Endocr., Paris*, **12**, 150.

RIESCO, A., AND SCHWARZ, J.—(1944) *Bol. Soc. Biol. Santiago de Chile* **1**, 69.

ROGERS, J. B.—(1951) *J. Geront.*, **6**, Suppl. 3, 142.—(1952) *Ibid.*, **7**, No. 3.

THUNG, P. J.—(1956) Experimental Research on Ageing, Symp., Basel. (Birkhäuser, Basel), p. 74.—(1958) 'Ovaria van oude muizen'. Thesis, University, Amsterdam, 1958.

Idem., BOOT, L. M., AND MÜHLBOCK, O.—(1956) *Acta Endocr., Copenhagen*, **23**, 8.

WARREN, S., AND GATES, O.—(1941) *Cancer Res.*, **1**, 65.

WOOLLEY, G. W.—(1950) *Recent. Progr. Hormone Res.*, **5**, 383.

ON THE OCCURRENCE AND MORPHOLOGY OF SPONTANEOUS TUMOURS IN THE PITUITARY, ADRENALS, GONADS AND MAMMARY GLANDS IN RATS BEFORE AND AFTER GONADECTOMY, IRRADIATION AND TREATMENT WITH OESTROGEN OR PROGESTERONE

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MUCH suggests that hormones play a complex role in the pathogenesis of mammary tumours. In a closed colony of rats kept at the Gynaecological Department, University Hospital, Lund, tumours often occur spontaneously in the mammae of the older animals. It was therefore decided to study the animals systematically for tumours in endocrine organs (pituitary, adrenals and gonads), which might have some relation to mammary tumours.

Since castration and treatment with oestrogen or progesterone are known to affect the development of mammary tumours in rats (Geschickter, 1947 ; Burrows and Horning, 1952), the influence of such measures was also studied.

Roentgen irradiation, which has a carcinogenic effect, was also included in the investigation.

MATERIAL AND METHODS

Virginal female rats and male rats that had since birth been kept separate from females, were observed, generally until their natural death (occasionally some of the animals were killed to provide material for histological examination). All the animals were raised alike in cages of the same size and on a diet of grain, milk and kitchen refuse.

Some male and female animals were gonadectomised at 3 weeks of age.

Two groups of spayed females received injections of either oestrogen (1·25 mg. oestradiolbenzoate in 0·25 c.c. oil) or progesterone (12·5 mg. progesterone in 0·25 c.c. oil) once a week from the age of 1 to 12 months.

A single X-ray dose over the whole body was given to some animals—intact and spayed females—when they were 3 weeks old. When combined with spaying the dose was given on the day after the operation. The " air dose " given was 300 r (filter 1 mm. Al, ½ mm. Cu ; distance 50 cm., exposure time 3·9 minutes).

The animals were examined post mortem for any tumours. In none of the animals were any tumours found in the organs studied before the age of 12 months. The animals that lived for more than 12 months are listed below.

Intact males	49
Intact females	125
Castrated males	44
Spayed females	98
Irradiated intact females	67
Irradiated spayed females	45
Oestrogen treated spayed females	48
Progesterone treated spayed females	48
Total	574

Tumours were fixed in 10 per cent formalin and embedded in paraffin. The microscopical sections were stained with haematoxylin-eosin.

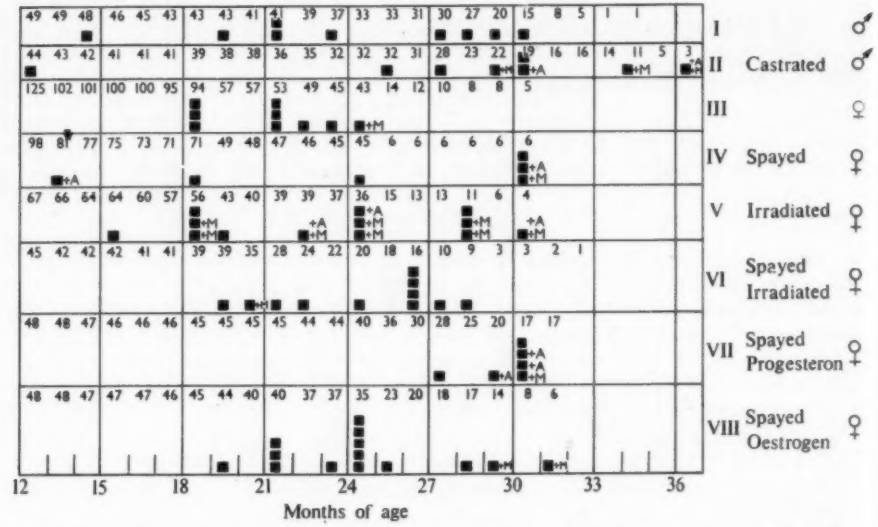


FIG. 1.—Hypophyseal tumours in different groups of rats. Each filled square indicates one rat dying from such a tumour. M and A designate co-existing tumours in the mammary glands and adrenals. The numerals give survivals in various age classes.

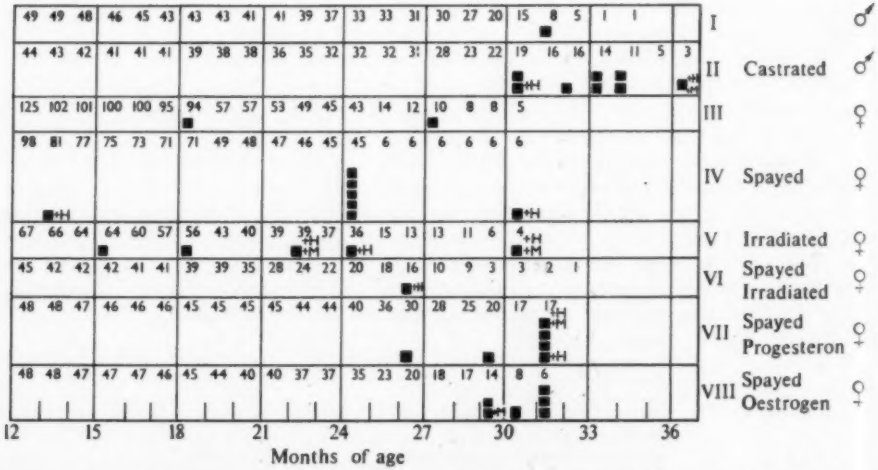


FIG. 2.—Adrenal tumours in different groups of rats. Each filled square indicates one rat dying from such a tumour. H and M indicate co-existing tumours in hypophysis and mammary glands. The numerals give the survivals in the various age classes.

RESULTS

Tumour frequency

The incidence of tumours in the pituitary, adrenals and mammae in the various groups is given graphically in Fig. 1, 2 and 3.

In all the groups the frequency increased markedly with the ages of the animals.

In the statistical analysis the expected tumour development was assessed by placing the tumour frequency in relation to the number of "risk months" (the number of animals surviving a number of months). These expected values were compared with the number observed.

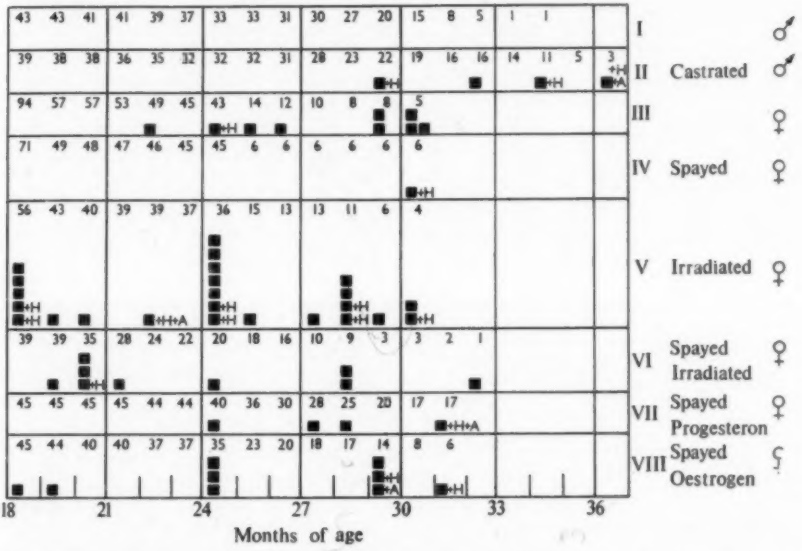


FIG. 3.—Mammary tumours in different groups of rats. Each filled square indicates one rat dying from such a tumour. H and A designate co-existing tumours in hypophysis and adrenals. The numerals give the survivals in the various age classes.

The expected and observed frequencies of tumour in spayed female rats with or without treatment with X-rays or progesterone—compared with intact females, intact and irradiated or spayed and oestrogen-treated—are given below:

	Pituitary tumour	Adrenal tumour	Mammary tumour
Number of observed occurrences	23	14	14
Calculated No. of occurrences	28·8	12·8	26·6
Difference	−5·8	+ 1·2	−12·6

The difference between the observed and expected values for mammary tumours was more than three times the mean error. The same tendency was found for the pituitary tumours, but the difference was only about 1·5 times the

mean error. The difference in frequency of mammary tumours showed the same trend in all the months and in all of the experimental groups.

For the pituitary tumours the negative difference was statistically significant only for the progesterone-treated spayed female rats during the observation period of 18–26 months compared with the group treated with oestrogen.

Irradiation produced a statistically significant increase in the incidence of pituitary and mammary tumours, but not of adrenal tumours in the combined groups of intact-spayed females compared with irradiated intact-spayed females.

Intact and spayed females, intact irradiated and spayed irradiated females, and oestrogen-treated and progesterone-treated spayed females were compared in the same way for mammary tumour frequency. The observed tumour frequency in each group was compared with the expected frequency. An analysis of this kind shows a statistically significant increase of the number of tumours in intact compared with spayed females—with or without irradiation—and in oestrogen-treated spayed females against progesterone-treated. The opposite kind of difference, though not statistically certain was found on comparison of intact and castrated male animals.

A point of interest is that some tumours occur in combinations too often to be ascribed to chance. Thus 4 animals had had all 3 (pituitary, adrenal and mammary) kinds of tumours. This coincidence is remarkable.

The combination of pituitary-mammary tumours occurred in all the various types of animals studied and to a greater extent than was theoretically expected :

	Observed number of animals with combined pituitary-mammary tumours	Expected number of animals with combined pituitary-mammary tumours
Intact and castrated males	3	0·7
Intact, spayed and spayed hormone-treated females	5	3·4
Irradiated females	9	6·8

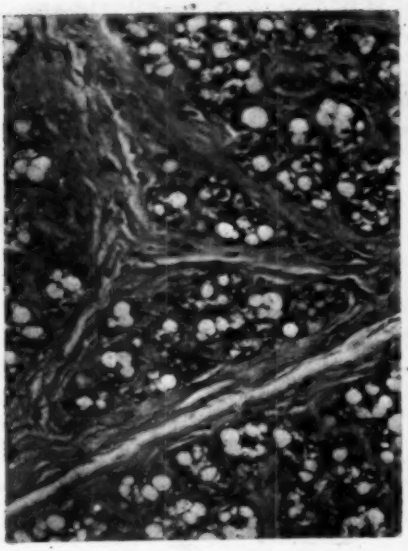
There was no such evidence of a concomitant, non-fortuitous coincidence of the combination adrenal tumour and mammary tumour.

On the other hand, the combination of pituitary and adrenal tumours revealed a preponderance that is also recorded in all the groups :

	Observed number of animals with combined pituitary-adrenal tumours	Expected number of animals with combined pituitary-adrenal tumours
Intact and castrated males	2	1·6
Intact, spayed and spayed hormone-treated females	5	3·1
Irradiated females	4	1·0

EXPLANATION OF PLATE

FIG. 4.—Mammary tumour (fibroadenoma) in a female rat. H and E.
FIG. 5.—Mammary tumour (sarcoma in a female rat. H and E.
FIG. 6.—Mammary tumour (adenoma) in an irradiated intact female rat. H and E.
FIG. 7.—Mammary tumour in an irradiated spayed female rat. H. and E.



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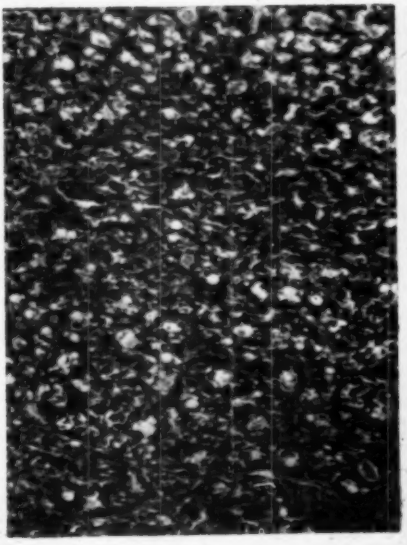

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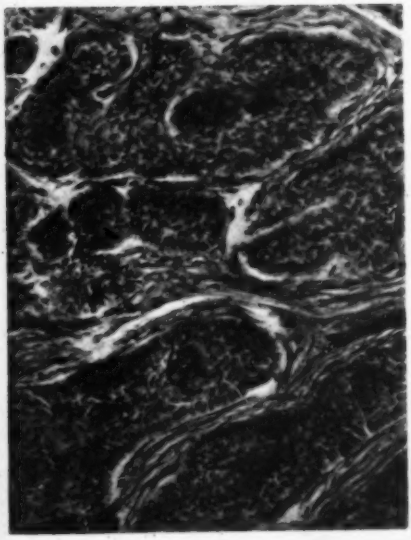

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Ovarian tumours were observed in 4 intact, non-irradiated females at the age of 2 years. These animals had no tumours in the pituitary, adrenals or mammae. A testicle tumour was seen in an intact male rat, aged 38 months. Moreover, one also occurred in a testis remnant in a " castrated " male which had reached the age of 36 months (all other gonadectomized animals—males and females—had no visible gonadal vestiges when examined post mortem).

Tumour morphology

All the *pituitary tumours* observed were bluish-red and richly vascularized. They varied in size : some were as large as a hazel-nut, while others were much smaller or even minute and were then varified microscopically. The tumours seemed to press the cerebral tissue aside without infiltrating it. Animals with pituitary tumours were either markedly restless and irritable or somnolent. Balance disturbances were observed. Microscopically the pituitary adenomata were composed almost entirely of large, pale, chromophobe cells with large, pale nuclei and usually very distinct nucleoli. Both basophilic and—more rarely—eosinophilic cells were sometimes present. Congestion of the capillaries, larger vessels and sinusoids was common.

The *adrenal tumours* were all cortical, protruding from the surface or so large that the normal morphology of the gland was no longer discernible. In such cases the gland seemed to be completely buried in the tumours. No metastases were observed. The tumours were smooth surfaced and circumscribed. The cut surface of the tumours was mottled yellowish-red with massive yellow areas and large blood-filled sinusoids. Microscopically the tumours consisted of cells which, in some sections, were relatively small with a moderate amount of cytoplasm and small, dark, usually round nuclei, and in other sections large with pale, fatty cytoplasm and somewhat larger and paler nuclei. In some cases the cells were arranged in trabeculae or garland-like, sometimes in tubule-like patterns. Stroma was sparse. Practically all of the adrenocortical tumours had cavities filled with a faintly stainable mucinous fluid and resembling those sometimes seen in the testicular and ovarian tumours of these animals. The tumours also contained capillaries and larger vessels as well as blood-filled sinusoidal cavities. The structure of the cortical tumours in the adrenals did not vary with the types of animals studied.

The *testicular tumour* which had growth in a testicle vestige of an incompletely castrated rat was the size of a lentil, rounded and mottled, yellowish-red cut surface. In the intact male rat with a gonad tumour the distal part of one testicle had been completely involved by a tumour which both macroscopically and microscopically was of the same character as the vestigial-testicular tumour. The testicle on the other side was small. Its tubules were atrophic. Microscopically the tumours contained cells and tissue structures resembling those observed in granulosa cell-androblastoma type tumours in the ovaries (see below) and those seen in the cortical tumours of the adrenals. Relatively small cells with rounded, dark nuclei and, in some sections, fatty, large and pale cells were arranged either irregularly or in trabeculae, sometimes tubule-like. Large sinusoidal cavities filled with blood or faintly stainable fluid were common. Four *ovarian tumours* were observed in intact female animals, but no tumours were found in those that were irradiated. They were all of granulosa cell-androblastoma type (Kullander, 1956).

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The *mammary tumours* observed were all well circumscribed and mobile. No metastases were seen. They ranged in size from that of a hazel-nut to that of a goose's egg. In some instances the skin over the tumour ulcerated owing to its being rubbed against the floor or walls of the cage.

The microscopic structure was the same in eight out of nine tumours observed in intact females : fibroadenoma with rich development of glandular parenchyma and with histological signs of secretion (Fig. 4). A milky fluid oozed from the cut surface of the tumours. One mammary tumour had the character of a sarcoma (Fig. 5).

Only one tumour was observed in spayed females. There was extremely little glandular parenchyma and the microscopic picture closely resembled that of a fibroma.

There were no mammary tumours among intact males. Two of the 4 mammary tumours observed in castrated males—3 cases in combination with pituitary tumours—showed the same microscopical picture as the fibroadenomata in intact females, whereas the other 2 resembled the fibroma in spayed females.

Some mammary tumours preponderantly of sarcoma type were seen in the oestrogen-treated spayed females. The cells were fusiform or rounded with pale, relatively abundant cytoplasm and large nuclei. Mitoses were common. Other tumours were fibroadenomata with signs of secretion.

Irradiated intact females had mostly fibroadenomata, histologically resembling those found in untreated, intact females. One mammary tumour was of sarcoma type. Some tumours resembled adenomata (Fig. 6).

Irradiated spayed females had several mammary tumours with solid groups of epithelial tumour cells in a scanty stroma, showing hardly any signs of secretion (Fig. 7).

DISCUSSION

Reports of pituitary tumours—chromophobe adenomata—in old rats are on record (Wolfe, Bryan and Wright, 1938 ; Saxton, 1941 ; Saxton and Graham, 1944). In the Yale strain a pituitary adenoma was hardly ever seen in rats younger than 400 days. After this age the frequency increased with age to about 60 per cent for male rats and 30 per cent for females older than 600 days (Saxton and Graham, 1944). In the present study the youngest animal in which a tumour was observed was 12 months old (Fig. 1). Pituitary tumours were equally common in both sexes. No certain difference was found between gonadectomised and intact animals—either males or females—in respect to the occurrence of pituitary tumours. Cessation of production of thyroid hormone (surgical or radiological thyroidectomy) may increase the incidence of pituitary tumours of chromophobe character in mice, probably with almost exclusive secretion of thyrotropin (Furth *et al.*, 1955). Some chromophobe pituitary adenomata in mice have been presumed to produce mainly ACTH. These tumours, growing after irradiation of the whole body, are thought to be the indirect result of damaged adrenals with reduced cortical hormone production (Furth, Gadsen and Upton, 1953). Ceased gonad hormone production in the rats in the present material did not seem to favour the development of pituitary tumours. The growth of pituitary tumours in female rats, both intact and spayed, however, was promoted by total body irradiation. Previously this has been observed only in intact mice (Upton and Furth, 1953, 1955).

Injection of oestrogens into rats is known to increase the frequency of pituitary adenomata (Burrows and Horning, 1952). In the present material oestradiol treatment of spayed rats induced more pituitary adenomata than progesterone-treatment. The injections of oestradiol, which was stopped 7 months before the first pituitary tumour appeared in this group of animals, seems to have started irreversible pre-tumorous changes in the pituitary at an early stage.

In the rats investigated the pituitary tumours appeared to develop somewhat earlier than the mammary tumours, besides which there was a statistically significant correlation between pituitary and mammary tumours. Morphological-functional disorders of the pituitary thus appear to affect the mammary glands and favour the growth of tumours in them. The existence of such a connection is also suggested by other observations. Mice with chromophobe pituitary adenomata acquire alveolar hyperplasia and distension of the mammary gland ducts, a change which can also be seen though less marked, in elderly female mice without pituitary tumours (Upton and Furth, 1955). In rats of the Albany strain mammary fibroadenomata are fairly common, but not in the Vanderbilt strain. The anterior lobe of the pituitary in the Albany strain contains a much larger number of chromophobe cells. This is also a characteristic of elderly animals. Albany animals thus present signs of accelerated ageing in the pituitary (Wolfe and Wright, 1943).

Adrenal tumours—usually with a later age maximum than the pituitary adenomata—also occurred in a definite connection with pituitary tumours in the rats in the present investigation and may thus be secondary to a primary pituitary disorder. Although all pituitary adenomata seemed to have a similar morphology with principally chromophobe cells, it is possible that they had a varying production of hormones: mainly prolactin when combined with mammary adenomata — predilectively ACTH and/or gonadotrophins when combined with adrenal tumours.

Gonadectomy increases the frequency of cortical tumours in the adrenals in both male and female rats (Houssay *et al.*, 1951). This could not be demonstrated with certainty in the present material. Tumours in the adrenal cortex of rats produce oestrogens, progesterone and androgens (Houssay, 1954). Among the spayed females with adrenal tumours there were indications of stimulation of the uterus. The production of hormones in these tumours, however, is not of such a quality or quantity as to stimulate the development of mammary tumours. There was no trace of a connection between adrenal and mammary tumours in any of the groups of this material.

Total body irradiation was not seen to increase the frequency of adrenal tumours in the females.

In this investigation neither oestradiol nor progesterone proved to have any influence on the occurrence of adrenal tumours. Ambrad-Dominguiez and Cardez (1952) state that administration of oestradiol to spayed rats inhibits the occurrence of adrenal tumours, whereas progesterone and deoxycorticosteron are ineffective.

Spontaneous, endocrinously active ovarian tumours in rats are rare, but can be induced experimentally by implantation of ovarian tissue into the spleen of gonadectomized animals (Kullander, 1956). Roentgen irradiation of intact female rats caused no tumours in the ovaries, nor were Drips and Ford (1932) able to induce ovarian tumours in rats by X-ray.

Leydig-cell tumours have been observed after implantation of testicle tissue in the spleen of castrated animals (Twombley, Meisel and Stout, 1949). Biskind and Biskind (1945), who also implanted testicular tissue in the spleen of castrates observed tumour growth in one. Its histological structure resembled what was observed in the 2 cases of spontaneous testicular tumour in the present investigation, i.e. granulosa cell-androblastoma tumour.

The mechanism of tumour induction in animals in which ovarian or testicular tissue had been implanted into the spleen at gonadectomy is assumed to be due to an increased gonadotrophic stimulation after the cessation of influence of pituitary antagonistic gonadal hormones. These hormones are largely inactivated in the liver. It is possible that a considerable reduction of the gonad parenchyma may lead to a similar hormonal imbalance, which would explain the appearance of tumour in the testicular fragment of the incompletely castrated rat.

It was noteworthy that morphologically—and possibly functionally—similar tumours occur spontaneously in these rats in the testicles and ovaries as well as in the adrenal cortex. This might suggest that similar cells—capable of forming tumours induced by pituitary stimulation—are to be found in the gonads and the adrenal cortex.

Administration of oestradiol to spayed females caused an increased number of mammary tumours, thus verifying earlier observations (Geschickter, 1947). Observations on transplanted mammary fibroadenomata in rats (Millar and Noble, 1954) have shown that such transplants grow more quickly in female hosts. If the male or female host is gonadectomized, the growth of the adenoma-transplant is increased or decreased, respectively. In accordance with earlier observations (Heiman, 1943) the effects of progesterone are not such as to increase the number of mammary tumours in rats.

Total body irradiation of both intact and spayed female rats resulted in a large number of mammary tumours. But Furth and Furth (1936) and Furth and Butterworth (1936), who irradiated mice at the age of 5–12 weeks with 200–400 r over the whole body, observed a falling frequency of mammary tumours. Passoneau, Brues and Hamilton (1953), however, have reported increased occurrence of fibroadenomata in the mammary glands of rats exposed to local skin irradiation.

SUMMARY

Analysis was made of the frequency and morphology of spontaneous tumours in the pituitary, adrenals, gonads and mammary glands of a closed colony of rats. Besides intact females and males the material included animals gonadectomized at the age of three weeks, intact and spayed females exposed to roentgen irradiation of the entire body at three weeks of age, as well as spayed females treated with oestrogen or progesterone from the age of one to twelve months.

1. Spaying was followed by a reduction in the occurrence of mammary tumours. Mammary tumours were observed in castrated but not in intact males.

2. Oestrogen-treated spayed females acquired a larger number of pituitary tumours than progesterone-treated.

3. Irradiation increased the occurrence of pituitary and mammary tumours in both intact and spayed females. Adrenal tumours did not increase in frequency, nor were any ovarian tumours observed after irradiation.

4. The microscopical picture of 2 testicular tumours resembled that of some

granulosa cell-androblastoma tumours found in the ovaries and of a number of tumours of the adrenal cortex.

5. The combination pituitary-mammary tumours and pituitary-adrenal tumours occurred among the various types of animals to an extent that cannot be explained as fortuitous coincidences.

Morphological-functional disorders in the pituitary in association with tumours in the adrenals, gonads and mammary glands in rats are discussed.

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REFERENCES

AMBRAD-DOMINGUEZ, N. AND CARDEZA, A. F.—(1952) *Rev. Soc. argent. Biol.*, **28**, 129.
BISKIND, M. S. AND BISKIND, G. R.—(1945) *Proc. Soc. exp. Biol. N.Y.*, **59**, 4.
BURROWS, H. AND HORNING, E. S.—(1952) Oestrogens and Neoplasia. Oxford (Blackwell).
DRIPS, D. G. AND FORD, F. A.—(1932) *Surg. Gynec. Obstet.*, **55**, 596.
FURTH, J. AND BUTTERWORTH, J. S.—(1936) *Amer. J. Cancer*, **28**, 66.
Idem, DENT, J. N., BURNETT, W. T. AND GADSEN, E. L.—(1955) *J. clin. Endocrin.*, **15**, 81.
Idem AND FURTH, O. B.—(1936) *Amer. J. Cancer*, **28**, 54.
Idem, GADSEN, E. L. AND UPTON, A. C.—(1953) *Proc. Soc. exp. Biol. N.Y.*, **84**, 253.
GESCHICKTER, Ch. F.—(1947) 'Diseases of the breast.' Baltimore. (Lippincott).
HEIMAN, J.—(1943) *Cancer Res.*, **3**, 65.
HOUSSAY, B. A.—(1954) *Congr. period int. Gynéc. Obstet.*
Idem, B. A., CARDEZA, A. F., PINTO, R. M. and BURGOS, M. H.—(1951) *C.R. Soc. Biol., Paris*, **145**, 1712.
KULLANDER, S.—(1956) *Diss. Univ. Lund.*
MILLAR, M. J. and NOBLE, R. L.—(1954) *Brit. J. Cancer*, **8**, 495.
PASSONEAU, J. V., BRUES, A. M. AND HAMILTON, K. A.—(1953) *Proc. Amer. Ass. Cancer Res.*, **1**, 41.
SAXTON, J. A.—(1941) *Cancer Res.*, **1**, 277.
Idem AND GRAHAM, J. B.—(1944) *Ibid.*, **4**, 168.
TWOMBLEY, G. H., MEISEL, D. AND STOUT, A. P.—(1949) *Cancer*, **2**, 884.
UPTON, A. C. AND FURTH, J. (1953) *Proc. Soc. exp. Biol. N.Y.*, **84**, 255.—(1955) *J. nat. Cancer Inst.*, **15**, 1005.
WOLFE, J. M. AND WRIGHT, A. W.—(1943) *Cancer Res.*, **3**, 497.
Idem, BRYAN, W. R. AND WRIGHT, A. W.—(1938) *Amer. J. Cancer*, **34**, 352.

SUBCUTANEOUS RAT CHLOROMAS AND THEIR MYELOPEROXIDASE CONTENT

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A NUMBER of reports on human chloroleukemia and chloroma have appeared in the literature and the findings have been summarized by Kandel (1937). Animal chloroleukemia has been occasionally observed (Engelbreth–Holm, 1942) but it was not until the work of Hall and Knocke (1938) that a chloroma was carried experimentally in mice. This tumour was subsequently employed in chemotherapeutic studies (Flory, Furth, Saxton and Reiner, 1943; Flory, Steinhardt and Furth, 1946). Oberling, Guérain and Guérain (1939) were the first to report successful transplantation of chloroleukemia in the rat. The induction of chloroleukemia in rats, and animal passage, was accomplished by Shay, Gruenstein, Marx and Glazer (1951), (Shay, Gruenstein, Harris and Glazer, 1952) by gastric instillation of methylcholanthrene. Several studies have been reported on rats bearing this tumor (Sparks *et al.*, 1953, 1954; Shay, Gruenstein and Harris, 1954; Schultz, Shay and Gruenstein, 1954). In these studies the tumor was carried by intraperitoneal inoculation of the blood or other material bearing the neoplastic cells, and the pathological state was associated with widespread invasion and infiltration of malignant tissue.

A rat chloroleukemia, detected at the Mound Laboratory (Monsanto Chemical Co.) by Miss Marietta Miller (Harris *et al*, 1958) and carried at the Argonne National Laboratory by Dr. Harvey Patt and co-workers (Patt, Jackson and Maloney, 1957), was the source of the tumors discussed in the present report. Our aim was to obtain localized, subcutaneous chloromas which would appear regularly and consistently, and to follow the level of myeloperoxidase activity and the associated morphological state of the tissue during progressive stages of growth. This aim was largely realized and the ensuing observations constitute a report of our results.

MATERIALS AND METHODS

The chloromas described below were derived from a chloromatous Sprague-Dawley rat which was inoculated intraperitoneally at the age of six weeks with 2 ml. of a tumor cell suspension containing 400×10^6 cells (Patt, private communication). The donor rat represented the tenth transfer into young adult rats and was designated as HP–C10, 2. When killed, 33 days after inoculation the donor rat showed the characteristic green infiltration and invasion throughout the abdominal mesentery, the pleural cavity, and the body wall. Large tumor masses were present in the body wall and in the region posterior to the liver. The

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latter mass was excised under sterile conditions to be used as donor tissue. This tissue was cut into pieces approximately 1 mm.^{3} in size which were implanted bilaterally into five Sprague-Dawley weanling rats with the conventional trocar technique. These rats were designated as HL–Cl, 1–5. Three animals developed bilateral tumors and one a unilateral tumor 17 days after implantation. Three of the seven takes regressed and the remaining four grew to large size. On the 60th day after implantation, one of the rats was killed and the tumor tissue was transplanted by trocar into 11 weanling rats, HL–C2, 1–11. Only two takes were obtained with latent periods of 38 and 49 days, respectively. The latter regressed and the former was used for transplantation into 11 two-day-old suckling rats, HL–C3, Group 1 (Table I). The suckling rats were lightly anesthetized and tumor bits were implanted with fine forceps, subcutaneously, in the dorsal thoracic region. The site of forceps entry was sealed with a drop of collodion. One of these recipients was taken 41 days after implantation, as donor, for the next transfer, HL–C4, Group 2. These and all subsequent implants were made by inserting an 18-gauge trocar near the base of the tail and implanting the tumor tissue in the dorsal cervical region. The transplantation data are summarized in Table I.

TABLE I.—*Chloroma Transplants in Sprague-Dawley Suckling Rats*

Group	Transplant generation	N	Mean suckling wt. (g.)	Number of takes	Percentage of takes	Mean latent period (days)	Remarks
1*	HL—C3	11	—	11	100	43	2 regressions
2	HL—C4	10	—	8	80	35	4 regressions
3	HL—C5	12	7·1	12	100	30	
4	HL—C5	12	—	11	92	36	2 regressions
5	HL—C5	11	7·8	10	91	30	1 regression
6	HL—C5	11	6·3	9	82	32	3 regressions
		67 ← Totals →		61	91 ← Mean →	35	

* Group 1, implanted with forceps technique; all other groups implanted with 18 gauge trocar.

In the harvesting of tumours the animals were killed by etherization, the tumors excised, and samples taken from opposite poles of each tumor. In many instances the tumor grew in chain-like fashion in an anterio-posterior direction along the needle track. With such growths samples were taken from each end of the chain and from the mid-portion. Imprints were made with the freshly cut surface of each sample after which the samples were stored in the dry-ice cabinet (−70° C.) until taken for myeloperoxidase (MPO) assay. The Papanicolaou stain was applied to imprints of all the specimens; imprints of 30 specimens were treated with the Wright-Giemsa and Washburn peroxidase stains (Lillie, 1954). No significant differences were observed between different regions of the tumor, and the figures presented represent the average of all samples from each tumor.

For MPO assay 10–30 mg. samples of each stored, frozen tissue specimen were taken in duplicate or triplicate, homogenized in a Virtis " 23 " homogenizer with 0·01 M tris buffer, pH 7·0, containing 0·1 per cent Triton-X-100; treated with n-butanol at a 5 per cent level for five minutes, made to one ml. final volume, centrifuged, and assayed with the guaiacol procedure using k_1 conditions (Colowick and Kaplan, 1955; Loeb and Doniger, 1958). Assays were performed at three dilutions for each tissue extract.

Dry weights were obtained, in duplicate, on all tissue samples by drying at 105° C. to constant weight.

RESULTS

Comparison of the findings for the tumors of the three transplant generations reveals no clear differences in the percentage of takes, the latent periods, or the incidence of regression (Table I). Under the conditions employed the percentage of takes was 91 with a mean latent period of 35 days. The tumors remained largely confined to the area of implant although some grew along the needle track. Such growths showed more adequate vascularization and less necrosis than the tumors which grew spherically at the implant site. Of the 21 takes in transplant generations C–3 and C–4 six regressions, 29 per cent, were observed. Of the 46 takes in the C–5 transplant generation six regressions, 13 per cent, occurred. Data on the regressed tumors are tabulated in Table IV. In another series of rats, which is not included with the tabulated data because of unsatisfactory donor tissue, the latent periods were longer and the regression rate was higher. Of 17 takes in this series, seven of the tumors, 41 per cent, regressed.

TABLE II.—*Data on Rat Chloromas (Mean and Standard Error)*

Group	N	Latent period (days)	MPO (" e " units per g. wet wt.)	Dry wt. of tumour %
1	9	43·0 ± 2·9	70·3 ± 3·9	19·6 ± 0·49
2	4	35·5 ± 1·1	69·3 ± 3·5	18·3 ± 0·23
3	12	30·0 ± 1·0	56·3 ± 2·9	18·6 ± 0·24
4	9	36·0 ± 2·0	55·3 ± 5·0	18·8 ± 0·32
5	9	30·0 ± 1·0	85·4 ± 6·8	18·3 ± 0·27
6	6	32·0 ± 2·2	66·0 ± 4·7	19·2 ± 0·24

The MPO activity of non-regressed tumors in transplant generations C–3 and C–4 (Table II) was essentially uniform with a mean value of 70 " e " units per gram of wet weight (Loeb and Doniger, 1958). Three purified preparations of myeloperoxidase from rat chloroma tissue which gave characteristic spectral absorption curves were assayed and found to have a spectral absorbance at 430 mμ of 0·05 for an activity of 1·0 " e " unit per ml. If we apply Agner's data (1958) on the $A_{430\ m\mu}$ of crystalline dog myeloperoxidase (1·2 for 1 mg. per ml.) to our preparations we obtain a value of 24·0 " e " units per mg. For activities of 70 " e " units per gram of wet weight of chloroma tissue, the MPO content would then be 0·29 per cent. The values for the C–5 transplant generation were somewhat lower except for Group 5 which contained three specimens showing high values of 109, 113, and 117 " e " units, respectively. The overall mean for Groups 3, 4, 5, and 6 is 65·8 " e " units per gram. The significance of this difference is questionable. No differences were found which could be related to the duration of the latent period, the period of growth, or the transplant generation. The mean value for the tumor dry weight, 18·8 per cent, was also essentially uniform for all samples. From Table III it is evident that there is no significant difference in the myeloperoxidase activity of the tumor tissue at progressive stages of growth. This is brought out more clearly by Fig. 1 which shows a plot of the mean total enzyme activity of the tumours against period of growth. Since the imprints

showed no difference in cell distribution related to the growth period, the linear relationship indicates that the enzyme-bearing cells are increasing in number in a fixed proportion to the total cell population as replication proceeds. In terms of tumor mass and myeloperoxidase activity the growth process represents a daily increment of 0·5 grams and 33 " e " units, respectively (Table III).

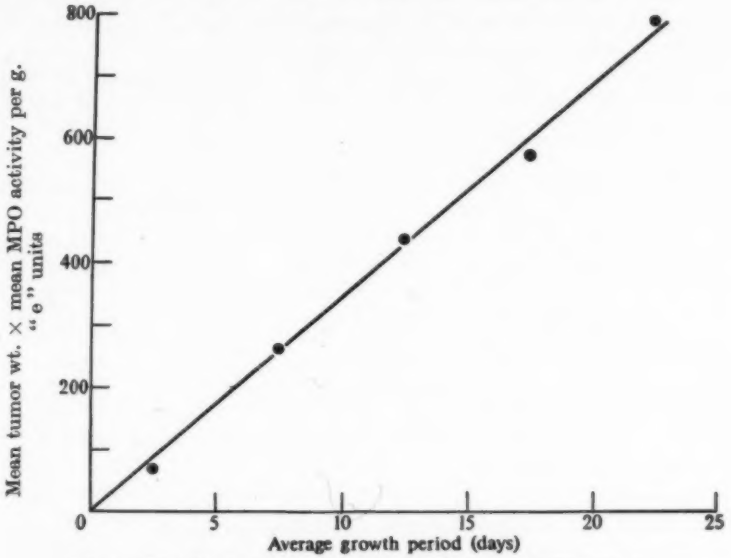

FIG. 1.—Rate of increase of total tumour MPO activity against time.

TABLE III.—*Tumour Weight and Myeloperoxidase Activity at Different Growth Periods (mean and standard error)*

Growth period (days)	N	Average tumour wt. (g.)	Average daily increment (g.)	Average MPO (" e " units per g. wet wt.)	Average MPO per tumour ("e" units)	Average daily increment ("e" units)
0–5	4	0·9 ± 1·5	0.36	74 ± 6·0	67	26·8
6–10	11	4·1 ± 0·71	0·55	63 ± 3·5	258	34·4
11–15	9	5·6 ± 0·53	0·45	77 ± 6·0	431	34·5
16–20	21	9·2 ± 0·83	0·53	62 ± 4·3	570	32·6
21–25	4	11·4 ± 1·14	0·51	69 ± 2·9	787	35·0
		Mean =	0·48			32·7

The regressed tumors are tabulated in Table IV. Some had regressed so far as to be impalpable but in these cases, at autopsy, a small pigmented area was observed at the tumor site. Only rats 128 and 129 in this series showed an increased latent period. The remaining 9 non-regressed tumors in this group showed a mean and standard error of 36 ± 2·0 days for the latent period. Prolonged latent periods were also observed in the group mentioned above which was implanted with donor tissue showing some necrosis. These observations suggest that the immune mechanism may be overwhelmed when the promotional stage of

tumor development preceeds at its normal rate, but may become dominant when promotion is retarded. Although tumor growth was not allowed to continue for prolonged periods in these experiments, necrotic alterations were apparent in many instances. This indicates that the immune response remains potentially functional with these tumors.

TABLE IV.—*Regressed Chloromas*

Group	Rat No.	Latent period (days)	Time of sacrifice* (days)	Tumour wt. (g.)	MPO ("e" units per g. wet wt.)	Dry wt. %
1	87	36	19	0·3	0	25·4
	92	37	6	—	—	—
2	102	35	19	2·8	39	17·7
	105	33	8	—	—	—
	106	35	13	1.3	43	20·2
	110	35	6	—	—	—
4	128	51	12	0·3	7	18·5
	129	51	24	0	—	—
5	144	27	18	0·7	47	20·2
6	151	32	16	0·3	12	20·6
	156	39	9	0·3	47	21·1
	159	27	21	2.1	46	17·7

Mean and standard error = 20·2 ± 0·82

* From end of latent period.

Histochemical observations brought out some interesting points, and raised some important questions. When normal rat bone marrow was stained with the Washburn peroxidase procedure the cells of the myeloid series fell into two groups on the basis of tinctorial properties ; in one group the cytoplasm showed a distinct brown color, in the other the color was neutrophilic. Virtually none of the segmented granulocytes showed the brown coloration. In the light of further observations discussed below, our own biochemical observations during purification of myeloperoxidase, and the findings of Bancroft and Elliot (1934) we have adopted the working hypothesis that MPO exists in two forms, one of which does not yield a positive histochemical response. Since two myeloperoxidase fractions can be demonstrated, based on ease of extractability from tissue, we postulate that this property may be related, in some way, to the histochemical response. The extracellular benzidine precipitation phenomenon (ECBP) in bone marrow, observed by Harris *et al.* (1958) and by us, may be due to the escape of the more " soluble " MPO from the cell. The customary positive or negative histochemical peroxidase reaction may depend, in some measure, on the degree of granule differentiation. It is tempting to consider the possibility that the brown coloration of the cytoplasm is due to the presence of the more " soluble " MPO derived from the granular material. After fixation one would not expect this " soluble " material to be removed during the straining process.

Imprints of a typical chloromatous tissue, when treated with the Wright-Giemsa, Papanicolaou and peroxidase stains, respectively, yielded the following observations : The Wright-Giemsa slides showed the characteristic primitive

cells of the myeloid series containing a large nucleus, with prominent nucleoli present in some. A preponderance of these cells showed cytoplasmic granules. With the Papanicolaou stain the granular character of the cells was more obscure, and the nucleoli were more abundant and distinct. Peroxidase stained imprints yielded a clearly negative peroxidase response, according to the usual criteria. Neither the dark cytoplasmic granules nor the brown coloration were observed. Direct assay of the tissue demonstrated a high degree of MPO activity so one must conclude that the activity occurs in a form which is not manifested histochemically. The positive response with peripheral blood or bone marrow smears is evidence that the negative reaction is not due to fixation or staining techniques.

DISCUSSION

The direct enzymatic assay of rat chloroma tissue for myeloperoxidase activity yielded remarkably uniform results. This is probably due, in part, to the fact that these tumors had been growing for a relatively short time so that secondary changes were at a minimum. This uniformity indicates that no significant cytological alteration occurred through the three transplant generations studied, or through the growth period considered. The careful study of Harris, Burke, Gruenstein and Shay (1958) indicates that no significant involvement of peripheral blood, bone marrow, or other reticuloendothelial tissue would be present in our animals. Our tumors were circumscribed. Invasion was limited to surrounding tissues and was not extensive. Other than the tumor, no gross pathology was observed at autopsy. The rats appeared lively and healthy throughout the experiment.

When implanted subcutaneously this tumor shows a definite tendency to undergo necrosis. If our tumors were allowed to grow for a longer period the regression rate would probably be considerably higher.

The negative benzidine-peroxidase test with tumor imprints is of particular interest. We agree with the suggestion of Harris *et al.* (1958) that the recipient organism may have some effect on the donor cells. More specifically, we would suggest that the interaction of leukemic cells with the reticulo-endothelial system may be essential to produce the peroxidase-positive histochemical reaction. If this could be demonstrated we should than have a clearer conception of the history of the leukemic cells found at a given site. One can conceive of several mechanisms to account for the peroxidase-positive reponse, from greater maturation and differentiation of the granules to structural alterations which would allow adsorption of myeloperoxidase on the surface of the granules.

The suggestion of Harris *et al.* (1958), that dehydration of paraffin-embedded tissues may inhibit the myeloperoxidase activity, seems unlikely. As Agner has reported (1941), this enzyme can withstand rather drastic treatment. Our observations suggest that the negative peroxidase response is due to the presence of the enzyme in a state which is not revealed by the histochemical technique employed.

These findings suggest caution in interpreting the histochemical test for myeloperoxidase. False negative results may well occur.

SUMMARY

A rat chloroma was carried by subcutaneous implantation in two-day-old suckling rats through three transplant generations. The myeloperoxidase activity

and dry weight of the tumors were determined at varying periods of tumor growth up to 25 days. Imprints were made of tumor specimens and these were treated with the Wright-Giemsa, Papanicolaou and Washburn peroxidase stains.

The myeloperoxidase activity and dry weight of these tumor tissues were quite uniform regardless of their size and transplant generation indicating no significant cytological change during the periods under study.

Tumor imprints stained with the Washburn peroxidase procedure gave a negative peroxidase response. It was postulated that the positive peroxidase response may depend upon interaction with the reticulo-endothelial system. The suggestion was made that some of the histochemical effects may be due to two forms of myeloperoxidase with differing " solubilities ".

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REFERENCES

AGNER, K.—(1941) *Acta physiol. scand.*, **2**, Suppl. VIII, 1.
Idem.—(1958) *Acta chem. scand.*, **12**, 89.
BANCROFT, G. AND ELLIOT, K. A. C.—(1934) *Biochem. J.*, **28**, 1911.
COLOWICK, S. P. and KAPLAN, N. O.—(1955) ' Methods in Enzymology.' New York (Academic Press, Inc.), p. 770.
ENGELBRETH-HOLM, J.—(1942) ' Spontaneous and Experimental Leukaemia in Animals.' Edinburgh (Oliver and Boyd).
FLORY, C. M., FURTH, J., SAXTON, J. A. JR., AND REINER, L.—(1943) *Cancer Res.*, **3**, 729.
Idem, STEINHARDT, I. D. AND FURTH, J.—(1946) *Blood*, **1**, 367.
HALL, J. W. AND KNOCKE, F. J.—(1938) *Amer. J. Path.*, **14**, 217.
HARRIS, C., BURKE, W. T., GRUENSTEIN, M. AND SHAY, H.—(1958) *Blood*, **13**, 162.
KANDEL, E. V.—(1937) *Arch. intern. Med.*, **59**, 691.
LILLIE, R.—(1954) ' Histopathologic Technic and Practical Histochemistry.' New York (Blakiston), p. 224.
LOEB, H. G. AND DONIGER, R.—(1958) *Brit. J. Cancer*, **12**, 669.
OBERLING, C., GUÉRAIN, M. AND GUÉRAIN, P.—(1939) *Bull. Ass. franç. Cancer*, **28**, 214.
PATT, H. M., JACKSON, E. M. and MALONEY, M. A.—(1957) *Proc. Amer. Ass. Cancer Res.*, **2**, 238.
SCHULTZ, J., SHAY, H. AND GRUENSTEIN, M.—(1954) *Cancer Res.*, **14**, 157.
SHAY, H., GRUENSTEIN, M., MARX, H. E. AND GLAZER, L.—(1951) *Ibid.*, **11**, 29.
Idem, GRUENSTEIN, M., HARRIS, C. AND GLAZER, L.—(1952) *Blood*, **7**, 613.
Idem, GRUENSTEIN, M. and HARRIS, C.—(1954) *J. nat. Cancer Inst.*, **15**, 463.
SPARKS, S. J., STEVENS, M. L., LANDES, M. J., HALLIDAY, S. L., MCKENZIE, D. AND WILLIAM, J. H.—(1953) *Blood*, **8**, 655.
Idem, WALSH, M. B., SEBASTIANELLI, L., STEVENS, M., LANDES, J., HALLIDAY, S. L. AND OLESON, J. J.—(1954) *Cancer Res.*, **14**, 753.

THE METABOLISM OF NEOPLASTIC TISSUES: OXIDATION OF FATTY ACIDS AND GLUCOSE BY ASCITES TUMOUR CELLS IN THE ABSENCE AND PRESENCE OF 2:4-DINITROPHENOL*†

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It is a well-known fact that the endogenous respiration of tumour slices is hardly, if at all, stimulated in the presence of glucose; in many cases even an inhibition of the oxygen consumption has been observed (Crabtree effect; Crabtree, 1929). The latter effect is especially pronounced in ascites tumour cells (Kun, Talalay and Williams-Ashman, 1951; Racker, 1956).

Following the early studies of Dickens and Simer (1930) on the respiratory quotients of tumours, it has become increasingly clear in recent years that fatty acid oxidation constitutes an important metabolic pathway in tumours. Thus, slices of solid tumours (Chapman, Brown, Chaikoff, Dauben and Fanash, 1954) and isolated tumour mitochondria (Emmelot and Bos, 1955*a*, 1955*b*, 1957; Emmelot, Bos, Brombacher and Hampe, 1959) have been found to oxidize added fatty acids, whereas solid and ascites tumours labelled *in vivo* with ^{14}C-palmitate, have been reported to liberate ^{14}C-carbon dioxide in appreciable quantity on incubation *in vitro* (Medes, Paden and Weinhouse, 1957). The respiratory quotients of ascites tumour incubated in the absence of oxidizable substrate is also indicative of the fact that fatty acid oxidation takes place (Slechta, Jakubovic and Sorm, 1955) and the shift in R_Q to unity, observed after addition of carbohydrate, suggests that the latter may replace the endogenous fatty acids in the terminal oxidations. Accordingly, in describing the Crabtree effect both the fatty acid oxidation cycle and the citric acid cycle (including the pyruvic dehydrogenase) should be taken into account. In a previous paper (Emmelot and van Vals, 1957) the effect of a high concentration of glucose on the oxidation of various ^{14}C-labelled substrates by ascites tumour cells has been reported. In the present investigation these experiments have been extended to include the effect of various glucose concentrations on the oxidation of acetate, butyrate, leucine and glutamate labelled with ^{14}C in carbon atom number one. The oxidation of glucose carbon has been traced by using uniformly ^{14}C-labelled glucose. Valuable information bearing on the oxidations of endogenous fatty acids and glucose has been published in the meantime by Bloch-Frankenthal and Weinhouse (1957) and Medes and Weinhouse (1958), who showed that the endogenous fatty acid oxidation was progressively decreased and that glucose carbon appeared in increasing amounts in the respiratory CO_2 when incubation was carried out in the presence

* The main results of this investigation were communicated at the 7th International Cancer Congress, London, July, 1958.

† Abbreviations are used as follows: P_i for inorganic phosphate; ADP and ATP for adenosine di- and triphosphate; DNP for 2:4-dinitrophenol.

of increasing concentrations of glucose. In the experiments of the latter authors the Crabtree effect was mainly exerted on the endogenous respiration. In most of our experiments the glucose carbon replaced the endogenous carbon much to the same extent, regardless of the amount of glucose present. Since DNP completely abolishes the Crabtree effect (Emmelot and Bos, 1959) it was also investigated whether the extra carbon dioxide produced under the latter condition was derived from endogenous substrate or from the added glucose.

MATERIALS AND METHODS

The S_3A ascites mammary carcinoma was maintained by serial transfer in $F_1(A \times C_3H)$ mice. The cells were harvested 7–8 days after inoculation. The suspension was diluted with the same volume of physiological saline. Since the suspension contained in most cases few erythrocytes, one low-speed centrifugation in the cold sufficed to obtain a colourless pellet.

The experiments were carried out in Krebs-Ringer phosphate buffer, containing 0·01, 0·02 or 0·03 M phosphate, at 37° C; 5–6 ml. buffer and 200–300 mg. wet weight of cells were used. The gas phase was 100 per cent oxygen; the duration of experiments as indicated; the respiratory carbon dioxide was collected as mentioned previously (Emmelot and Bosch, 1955). The ^{14}C-labelled substrates were either synthesized or obtained from the Radiochemical Centre, Amersham. 0·3–1·0 μ mole of [1–^{14}C] leucine and [1–^{14}C] glutamate were used.

The specific activity (SA) of the respiratory CO_2 is expressed as counts per minute of $BaCO_3$ at "infinite thickness". The relative specific activity (RSA) of the CO_2 is calculated as: (SA of respiratory CO_2/SA of ^{14}C-substrate as $BaCO_3$) $\times$ 100.

RESULTS AND DISCUSSION

The effect of glucose on the oxidation of [1–^{14}C] *acetate and* [1–^{14}C] *butyrate*

The oxidation of [1–^{14}C] acetate was not inhibited in the presence of glucose (0·5, 1 and 3 mg. glucose/flask containing 6 ml. Krebs-Ringer phosphate buffer of 0·01 or 0·02 M) during 60 minutes incubation at 37° C. A stimulation of acetate oxidation (up to 50 per cent) at medium glucose concentrations and a slight inhibition at 6 mg. glucose/flask was consistently observed (compare also Emmelot and van Vals, 1957). A typical experiment is listed in Table I; the SA of the carbon dioxide, produced in the absence of glucose, amounted to approximately 1 per cent.

The oxidation of [1–^{14}C] butyrate, however, was markedly depressed, *i.e.* for 45, 57 and 68 per cent, in the presence of 1, 3 and 6 mg. glucose/flask, respectively. No difference in results was obtained in a number of parallel experiments ran in 0·01 and 0·02 M phosphate buffer (Table I). The effect of glucose on the oxidation of [1–^{14}C] butyrate should have been the same as on that of [1–^{14}C] acetate if butyrate had been oxidized in the presence of glucose first by the fatty acid oxidation cycle to 2 molecules of "acetate". The fall in the SA of the CO_2 from [1–^{14}C] butyrate therefore suggests that the fatty acid oxidation cycle was inhibited in the presence of glucose. The inhibition of the oxidation of added butyrate, like that of the endogenous fatty acids (Medes and Weinhouse, 1958) occurs already at low glucose concentrations which do not give rise to the Crabtree effect. This phenomenon may be due to a competition between the pyruvic

dehydrogenase and the enzymes of the fatty acid oxidative cycle for diphosphopyridine nucleotide and coenzyme A.

Since glucose carbon also contributes to the respiratory CO_2 it was of interest to measure the conversion of [U–^{14}C] glucose to $^{14}CO_2$.

TABLE I.—*Effect of Glucose on the Oxidation of* [1–^{14}C] *Acetate and* [1–^{14}C] *Butyrate by S_3A Ascites Carcinoma Cells*

200 mg. of wet weight cells incubated during 60 minutes in 6·0 ml. Krebs-Ringer phosphate buffer (in Experiments 1 and 2 : 0·02 M ; in Experiment 3 : 0·01 M phosphate), 1·5 mg. labelled fatty acid added as sodium salt. The RSA of the respiratory CO_2 amounted to approximately 1 per cent.

		Carbon dioxide		
Substrate	Glucose (mg./flask)	Amount (μ moles) (a)	SA (c./m.) (b)	Total counts/min. ($a \times b$)
[1–^{14}C] acetate	0	20·5	3,080	63,140
	0·5	22·0	2,772	60,984
	1·0	20·5	4,320	88,560
	3·0	18·5	3,854	71,299
	6·0	15·0	3,750	56,250
[1–^{14}C] butyrate	0	22·0	1,902	41,844
	1·0	23·0	989	22,747
	3·0	20·0	965	19,300
	6·0	17·5	786	13,755
[1–^{14}C] butyrate	0	21·5	1,920	41,280
	1·0	21·5	1,077	23,156
	3·0	20·0	828	16,560
	6·0	16·5	800	13,200

The oxidation of [*U*–^{14}*C*] *glucose*

The production of $^{14}CO_2$ from various concentrations of [U–^{14}C] glucose by the ascites cells was studied under the same conditions as mentioned above. No essential differences were observed in the results of experiments carried out in the 0·01 or 0·02 M phosphate buffer. At low glucose concentrations which did not depress the carbon dioxide production below the endogenous level, glucose carbon (^{14}C-glucose → ^{14}C-pyruvate → ^{14}C-acetyl-CoA and ^{14}C-oxalacetate) appeared in the respiratory carbon dioxide to a marked extent (Table II). At higher glucose concentrations the production of carbon dioxide decreased but the RSA of the CO_2 increased, so that the microatoms of glucose converted to carbon dioxide remained of the same order. Thus, under the present conditions the oxidation of glucose was almost independent of its concentration. The Crabtree effect was mainly exerted upon the oxidation of endogenous metabolites (compare also Medes and Weinhouse, 1958). Experiment 2 of Table II illustrates this particularly well ; most of our results were, however, more like that illustrated in the first experiment of Table II.

It is evident, then, that the oxidation of carbohydrate cannot substitute for the depressed oxidation of endogenous substrate, *i.e.* the amount of acetyl groups derived from excess glucose and endogenous metabolites and oxidized to CO_2, is smaller than that derived from endogenous metabolites in the absence of glucose. It must therefore be concluded that the Krebs cycle enzymes and the pyruvic

dehydrogenase are inhibited in the presence of excess glucose. That glucose does not inhibit the oxidation of [1–^{14}C] acetate may be due to the fact that glycolytic ATP actually increases the conversion of acetate to acetyl-coenzyme A; this may explain the rise in the specific activity in the carbon dioxide observed in the presence of glucose. Butyrate activation might be likewise enhanced but in that case its oxidation to 2 molecules of acetyl-coenzyme A would require extra ADP and P_i, which may be considered as the factor limiting the oxidative response in the presence of excess glucose (apart from the possibility of competition between coenzymes as mentioned above).

TABLE II.—*The Oxidation of [U–^{14}C] Glucose by S_3A Ascites Carcinoma Cells*

200 mg. of wet weight cells incubated during 60 minutes in 6 ml. Krebs-Ringer phosphate (0·02 M) buffer.

Glucose (mg./flask)	Carbon dioxide: μ moles (*a*)	Carbon dioxide: RSA (*b*)	Carbon dioxide: μ atoms glucose carbon oxidized ($a \times b$)/100
0	22·0	0	0
0·5	23·5	31	7·3
1·0	22·5	36	8·1
2·5	21·0	38	8·0
5·0	19·5	39	7·6
10·0	17·0	43	7·3
0	19·0	0	0
0·5	21·0	21	4·4
1·0	19·0	30	5·7
2·5	18·0	40	7·2
5·0	15·0	43	6·5
10·0	11·0	63	6·9

The effect of 2:4-dinitrophenol on the oxidation of [1–^{14}C] acetate in the absence and presence of glucose

[1–^{14}C] Acetate must be converted to acetyl-CoA before it can be oxidized by the ascites cells. This conversion is dependent upon ATP and, in the absence of carbohydrate, exclusively upon mitochondrial ATP. Since DNP abolishes the mitochondrial ATP synthesis, it may be expected that the uncoupling agent will inhibit acetate metabolism.

5×10^{-5} M DNP markedly inhibited the incorporation of ^{14}C from radio-acetate into the respiratory carbon dioxide of two ascites tumours (Table III) incubated for 150 minutes. The finding that the labelling of the proteins and the fatty acids was also markedly impaired favours the assumption that the formation of acetyl-CoA was blocked in the presence of DNP When the incubation was carried out under different conditions (Table IV) a similar effect was noted with 10^{-4} and 8×10^{-5} M DNP, but 5×10^{-5} M DNP was now less active. With 10^{-5} M and lower concentrations of DNP the oxidation of acetate was even slightly enhanced over the no-DNP level. Addition of glucose markedly counteracted the inhibitory effect of DNP with regard to the appearance of the tracer in both the CO_2 and proteins. This may indicate that the glycolytic ATP had a ready access to the acetate-activating enzyme.

TABLE III.—*Effect of DNP on the Metabolism of* [1–^{14}C] *Acetate by* S_3A *Ascites Carcinoma and Ascites Rhabdomyosarcoma Cells*

750 mg. of wet weight ascites rhabdomyosarcoma- and S_3A carcinoma cells, respectively, were incubated during 180 minutes in 2·5 ml. Krebs-Ringer phosphate (0·01 M) buffer in the presence of 1·25 mg. ^{14}C-labelled sodium acetate; $5 \cdot 10^{-5}$ M DNP added as indicated.

DNP	Carbon dioxide: μ moles	Carbon dioxide: μ moles acetate incorporated	Proteins (c./m.)	Fatty acids μ moles acetate incorp. $\times 10^3$
–	92·0	1·13	280	1·13
+	93·5	0·67	78	0·23
–	58·0	0·20	127	0·14
+	62·5	0·11	53	0·07

TABLE IV.—*Effect of DNP on the Oxidation of* [1–^{14}C] *Acetate by* S_3A *Ascites Carcinoma Cells Incubated in the Absence and Presence of Unlabelled Glucose*

150 or 200 mg. of wet weight cells were incubated during 60 minutes in 6 ml. Krebs-Ringer phosphate (0·02 M) buffer in the presence of 1 mg. Na-(1–^{14}C) acetate. The SA of the radioacetate used in the first three experiments was half that used in the other experiments. Glucose (6 mg.) added as indicated.

DNP (molar)	Glucose	Carbon dioxide: Amount (μ moles) (*a*)	Carbon dioxide: SA (c./m.) (*b*)	Carbon dioxide: Radiochemical yield (c./m.) ($a \times b$)	Proteins (c./m.)
..	–	20	850	17,000	–
10^{-4}	–	20·5	332	6,806	–
$5 \cdot 10^{-5}$	–	22·5	768	17,280	–
10^{-5}	–	20·5	984	20,172	–
..	–	20	1,260	25,200	–
$8 \cdot 10^{-5}$	–	21·5	457	9,826	–
$4 \cdot 10^{-5}$	–	23·0	832	19,136	–
$8 \cdot 10^{-6}$	–	20	1,455	29,100	–
..	–	10·5	1,035	10,867	20
10^{-4}	–	10	518	5,180	5
10^{-4}	+	20	1,025	20,500	42
10^{-4}	–	10	1,724	17,240	–
10^{-4}	+	16	2,315	37,040	–
..	–	10	3,100	31,000	50
10^{-4}	–	10	1,908	19,080	29
10^{-4}	+	16	2,400	38,400	60

The effect of 2:4-dinitrophenol on the oxidation of [*U*–^{14}C] *glucose and endogenous metabolites*

The inhibition of the respiration following addition of excess glucose to ascites tumour cells can be completely eliminated by DNP (compare Emmelot and Bos, 1959). In view of the fact that the ascites cells oxidize both endogenous and glucose carbon, it was of interest to know whether DNP stimulated the oxidation of the endogenous substrate—fatty acids, according to Medes and Weinhouse (1958)—or that of the added carbohydrate.

The effect of DNP was studied on cells incubated in the presence of such a concentration of [U–^{14}C] glucose that the respiration and carbon dioxide production were markedly depressed. In view of an inhibitory effect of DNP which may develop as a result of the pH change of medium following the increased aerobic glycolysis (Emmelot and Bos, 1959) short periods of incubation, relatively small amounts of cells and a 0·02 M phosphate buffer were used. The marked rise in the RSA of the respiratory carbon dioxide observed in the presence of DNP (Table V) indicates that primarily the oxidations which converted the glucose-derived pyruvate to carbon dioxide were enhanced. Simple calculation showed that the extra carbon dioxide evolved in the presence of DNP is mainly or completely derived from glucose carbon (*e.g.* in the first experiment of Table V: in the presence of 10^{-4} M DNP 17·5-10 = 7·5 μ atoms C extra converted to CO_2 of which 7·9–1·2 = 6·7 μ atoms were derived from glucose-C).

TABLE V.—*Effect of DNP on the Oxidation of [U–^{14}C] Glucose by S_3A Ascites Carcinoma Cells*

200 mg. of wet weight cells suspended in Krebs-Ringer phosphate (0·02 M) buffer of pH 7·4. The experiment marked (*) was carried out with 700 mg. wet weight of rhabdomyosarcoma ascites cells

Glucose (mg.%)	Time of incubation (minutes)	DNP (molar)	Carbon dioxide: Amount (μ moles) (*a*)	Carbon dioxide: Relative SA (*b*)	Carbon dioxide: Glucose carbon oxidation (μ atoms) ($a \times b$)/100
112	35	..	10	12	1·2
(7 mg./6 ml. medium)		10^{-4}	17·5	45	7·9
		$5 \cdot 10^{-5}$	22·5	54	12·2
		10^{-5}	15	38	5·7
100	45	..	12·5	17	2·1
(5 mg./5 ml. medium)		$8 \cdot 10^{-5}$	24	42	10·1
		$4 \cdot 10^{-5}$	20	50	10·0
		$8 \cdot 10^{-6}$	16·7	32	5·3
167	35	..	10	27	2·7
(10 mg./6 ml. medium)		$5 \cdot 10^{-5}$	18	44	7·9
	60	..	16	34	5·4
		$5 \cdot 10^{-5}$	21	54	11·3
50(*)	150	..	57	34	19·4
(1·5 mg./3 ml. medium)		$5 \cdot 10^{-5}$	85	45	38·3

The reason for the failure to stimulate the oxidation of endogenous substrates in the presence of DNP and excess glucose is not necessarily due to an inhibition of the fatty acid oxidation cycle by DNP but may be due to a competition for coenzymes (diphosphopyridine nucleotide, coenzyme A), as discussed above.

The effect of glucose and 2:4-dinitrophenol on the oxidation of [1–^{14}C] leucine and [1–^{14}C] glutamate

It has been reported earlier (Emmelot and van Vals, 1957) that glucose depressed the oxidation of [1–^{14}C] leucine and increased that of [1–^{14}C] glutamate to carbon dioxide. The latter experiments were conducted under conditions in

which a marked pH change of the medium was likely to occur. It has now been found that the oxidation of the two amino acids by the ascites tumour cell is markedly dependent upon the pH. At pH values from 7·4 to 6·0 the oxidation of glutamate was progressively increased whereas that of leucine was decreased. Correcting for the limited pH change which occurred under the conditions of the present experiments (0·02 and 0·03 M phosphate buffer) it was found that glucose (0·006 M) inhibited leucine oxidation for about 20 per cent but had little effect on glutamate oxidation. The fall in protein labelling from [1–^{14}C] acetate by glucose observed earlier (Emmelot and van Vals, 1957) might thus have been due to a diminished introduction of ^{14}C into glutamate as a result of the effect of pH on the equilibrium [1–^{14}C]-α-ketoglutarate $\rightleftharpoons$ (^{14}C) glutamate.

DNP (10^{-4} M) stimulated leucine oxidation 30–75 per cent but depressed glutamate oxidation by 20–40 per cent in the absence of glucose; these effects were primarily due to changes in the SA but not in the amount of the CO_2 produced. These results show that DNP may affect the oxidation of the two amino acids differently.

SUMMARY

A study of the oxidation of acetate, butyrate, leucine and glutamate, all labelled with ^{14}C in carbon atom number one, to respiratory carbon dioxide by the S_3A ascites mammary carcinoma in the absence and presence of glucose and/or DNP, and of the oxidation of various concentrations of [U–^{14}C] glucose in the presence and absence of DNP, has shown the following results: the fatty acid oxidation cycle is impaired but the oxidation of acetate is not impaired by the presence of glucose; glycolytic ATP may substitute for mitochondrial ATP in the acetate-activating reaction; only a fixed amount of glucose is oxidized by the ascites cells under the present experimental conditions, regardless of the concentration of glucose; the extra CO_2 which is produced in the presence of DNP and excess glucose is mainly or exclusively derived from glucose; leucine and glutamate oxidation are affected differently both by DNP and by changes in the pH of the medium.

REFERENCES

Bloch-Frankenthal, L. and Weinhouse, S.—(1957) *Cancer Res.*, **17**, 1082.
Chapman, D. P., Brown, Jr., G. W., Chaikoff, I. L., Dauben, W. G. and Fanash, N. O.—(1954) *Ibid.*, **14**, 372.
Crabtree, H. G.—(1929) *Biochem. J.*, **23**, 536.
Dickens, F. and Simer, F.—(1930) *Ibid.*, **24**, 1301.
Emmelot, P. and Bos, C. J.—(1955*a*) *Rec. Trav. chim. Pays-Bas*, **74**, 1343.—(1955*b*) *Experientia*, **11**, 353.—(1957) *Enzymologia*, **18**, 179.—(1959) *Brit. J. Cancer*, **13**, 520.
Idem, Bos, C. J., Brombacher, P. J. and Hampe, J. F.—(1959) *Ibid.*, **13**, 348.
Idem and Bosch, L.—(1955) *Ibid.*, **9**, 327.
Idem and van Vals, G. H.—(1957) *Ibid.*, **11**, 620.
Kun, E., Talalay, P. and Williams-Ashman, H. G.—(1951) *Cancer Res.*, **11**, 855.
Medes, G., Paden, G. and Weinhouse, S.—(1957) *Ibid.*, **17**, 127.
Idem and Weinhouse, S.—(1958) *Ibid.*, **18**, 352.
Racker, E.—(1956) *Ann. N.Y. Acad. Sci.*, **63**, 1017.
Slechta, L., Jakubovic, A. and Sorm, F.—(1955) 'Resumées des Communications, 3ième Congèrs International de Biochimie.' (Bruxelles, 1955) p. 126 (14–39).

THE METABOLISM OF NEOPLASTIC TISSUES: THE EFFECT OF 2:4-DINITROPHENOL ON THE RESPIRATION OF ASCITES TUMOUR CELLS*†

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ADDITION of glucose to respiring ascites tumour cells is known to inhibit the endogenous oxidation (Kun, Talalay and Williams-Ashman, 1951; Racker 1956). This phenomenon is called the Crabtree effect (Crabtree, 1929). The inhibition of the respiration by glucose may be considered to represent the reverse situation of the inhibition of glycolysis by oxygen (Pasteur effect). Just as much as the inhibition of glycolysis is not caused by the oxygen *per se* but by the respiratory processes, so it seems that the inhibition of respiration is not due to glucose *per se* but to the glycolytic reactions. Whether also the same basic regulatory mechanism is involved is another question. After it was found that glycolysis and respiration were both dependent upon P_i and ADP, the former process because of the substrate phosphorylations and the latter because of the phosphorylations in the mitochondrial respiratory chain which appeared to be coupled to the oxidations, the possibility of a competition between the two processes was recognized (Johnson, 1941; Lynen, 1941, 1956, 1958). It is now generally assumed that the mitochondrial phosphorylations deprive the cytoplasm of P_i and ADP because the requirement for the latter compounds is very much higher in the case of the mitochondrial than of the glycolytic phosphorylations. This results in an inhibition of glycolysis (Pasteur effect) and of the triosephosphate dehydrogenase system in particular as compared with the situation under anaerobic conditions. It has recently been discussed (Chance and Hess, 1956; Racker, 1956; Lynen, 1956, 1958) that another effect may be superimposed upon that described above. ATP formed in the mitochondria, as a result of the oxidative phosphorylations, might not readily equilibrate with the cytoplasm and thus retard the ATP-dependent phosphorylation of glucose as compared with the situation under anaerobic conditions in which ATP is exclusively synthesized by the glycolytic reactions in the cell sap. This would explain the decreased glucose utilisation under aerobic conditions.

The above considerations allow the conclusion (Racker, 1956) that the reverse of the Pasteur effect, *i.e.* an inhibition of respiration by glycolysis (Crabtree effect) might also occur through the same basic mechanism and especially in those tissues in which, for some reason or another, the aerobic glycolysis is very high, as for instance in the ascites tumour cell.

The finding of Loomis and Lipmann (1948) that DNP uncouples the oxidations from the phosphorylations in isolated liver mitochondria, has contributed markedly

* The main results of this investigation were communicated at the 7th International Cancer Congress, London, July, 1958.

† Abbreviations are used as follows: P_i for inorganic phosphate; AMP, ADP and ATP for adenosine mono-, di- and triphosphate; DNP for 2:4-dinitrophenol.

to the formulation of the mechanism of the Pasteur effect in terms of a competition between glycolysis and respiration for P_i and adenine nucleotides. Since the rate-limiting mitochondrial phosphorylations are uncoupled from the oxidations by DNP, they no longer limit the oxidations, which then proceed at an increased rate. Moreover, the glycolytic reactions may now dispose of the greater part of the cellular P_i and adenine nucleotides, which are no longer required in the mitochondria. Accordingly, in the presence of DNP the aerobic glycolysis may reach the anaerobic level.

Kun *et al.* (1951) were the first to report that the Crabtree effect in the Ehrlich ascites carcinoma was counteracted by DNP. This finding has been substantiated by others (Racker, 1956; Seelich, Weigert and Letnansky, 1956; Kvamme, 1958; Ibsen, Coe and McKee, 1958). However, enhancement of the oxidation is not the only effect of DNP on the respiratory process of normal cells. Inhibition of the oxidation may also occur at higher concentrations of the uncoupling agent. The mechanism of this effect is not known. It has been suggested that the self-perpetuation of oxidative activity is dependent upon the presence of a certain amount of ATP and that, if the ATP concentration falls below a certain limit, an inhibition of respiration results; some support for this conclusion can be derived from studies with isolated mitochondria (Emmelot and Bos, 1957) and homogenates (Potter and Lyle, 1951). The optimal conditions for DNP to display the stimulatory effect on the respiration of glucose-supplemented ascites cells have as yet not been determined. Most authors report that the Crabtree effect is counteracted or abolished by DNP, but in many cases it is evident from the published data (compare, for instance, Seelich *et al.*, 1956) that after a stimulation of oxygen consumption a phase of inhibition occurs. This phenomenon has received some attention in the present investigation, and it will be shown that, under properly chosen conditions, it is possible to enhance the oxygen consumption of glucose-supplemented ascites cells by DNP to well over the endogenous level.

The present investigations included a study of the effect of DNP on the endogenous respiration, on the respiration in the presence of citric acid cycle intermediates and low glucose concentrations, and of the effect of the pH on the various oxidations. That the competition between respiration and glycolysis occurs at the level of the phosphorylations, as suggested by the effect of DNP, was shown more directly by the effect of citric acid cycle intermediates on respiration and glycolysis in the presence of excess glucose.

MATERIALS AND METHODS

The S_3A ascites mammary carcinoma was used in the present investigations (Compare Emmelot and Nout, 1959). The cells (dry weight 6–9·5 mg.) were suspended in 1·6 ml. Krebs-Ringer phosphate buffer, which contained 0·01, 0·02 or 0·03 M phosphate of pH 7·4. Incubation at 37° C. with air as gas phase. Oxygen consumption was measured by the ordinary Warburg technique. Lactate was determined according to Barker (1957).

RESULTS AND DISCUSSION

The effect of 2:4-dinitrophenol on the endogenous respiration

The endogenous respiration of S_3A ascites carcinoma cells, incubated in 1·6 ml. Krebs-Ringer phosphate buffer (0·01 M) of pH 7·4 at 37° C. for 60–180

minutes, was only slightly stimulated by DNP in the concentration range of $1–10 \times 10^{-5}$ M; in some exceptional cases a marked inhibition was found (compare below). The maximal stimulation was usually observed with 5×10^{-5} M DNP; it amounted to 25 per cent of the O_2 consumption of the controls. In the various experiments the effect of 10^{-4} M DNP varied from slightly inhibitory to slightly stimulatory. One of the experiments carried out with 10^{-4}, 5×10^{-5} and 10^{-5} M DNP is illustrated in Fig. 1; the Q_{O_2} of the control amounted to 9·3.

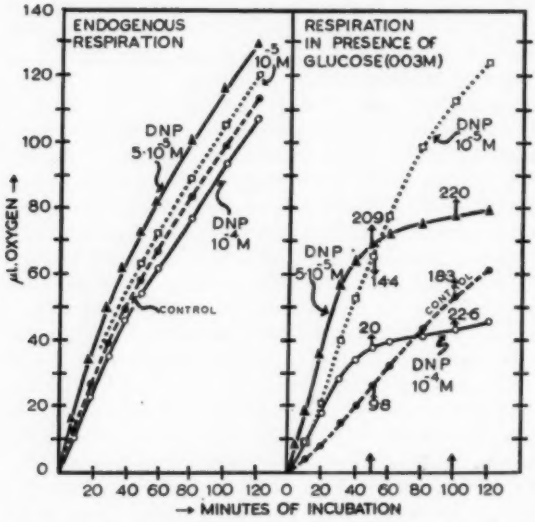

FIG. 1.—The effect of 2 : 4-dinitrophenol on the respiration of S_3A ascites carcinoma cells in the absence and presence of excess glucose.

Incubation in 1·6 ml. Krebs-Ringer phosphate (0·01 M) buffer of pH 7·4. Figures at 50 and 100 minutes denote µmoles of lactate produced. 6·3 mg. of dry wieght cells; 48 µmoles glucose present as indicated.

Since the endogenous oxidation of the ascites cells was not markedly enhanced by DNP, it might follow that in the absence of the uncoupling agent the mitochondrial oxidations were hardly, if at all, coupled with phosphorylations and already taking place at maximal or near-maximal rate. However, since in the absence of glucose the ascites cells oxidize endogenous fatty acids (Medes and Weinhouse, 1958) by way of the fatty acid oxidation cycle to acetyl-CoA and the latter to CO_2 via the citric acid cycle, and since it can be shown that the citric acid cycle oxidations are very markedly enhanced by DNP, it may be concluded that the lack of any marked effect of DNP on the endogenous respiration was due to a lack of oxidizable substrate or even to an inhibition by DNP of the oxidations. The O_2 uptake which is actually recorded in the presence of DNP (10^{-4} M) might thus be the result of two effects of DNP, *i.e.* an inhibition of part of the oxidation and a stimulation of the oxidation (due to uncoupling) of those metabolites which are still converted by the fatty acid oxidation and citric acid cycles.

Effect of 2:4-dinitrophenol on the respiration in the presence of excess glucose

Addition of glucose (48 μmoles, 0·03 M final concentration) to the ascites cells reduced the O_2 consumption to nearly half that of the endogenous level (Q_{O_2} in the presence of glucose = 5·0, Fig. 1). In the presence of 10^{-4} M DNP the respiration was enhanced initially, a 2-fold stimulation being noted after 30 minutes of incubation. The rate of respiration slowed down on further incubation so that after another 15 minutes the O_2-uptake had virtually ceased. The respiratory rate of the cells in the presence of glucose alone did not change and after a total of 80 minutes it surpassed that of the DNP-supplemented cells. The early stimulation obtained with 5×10^{-5} M DNP was much more pronounced than that induced by 10^{-4} M DNP but in the former case the respiration also levelled off after prolonged incubation. With the lowest DNP concentration (10^{-5} M) the respiratory rate was markedly enhanced during the whole period of incubation. The results show that under the present conditions DNP may abolish the Crabtree effect completely, but that the respiration may become impaired at some later stage during incubation with the uncoupling agent.

Measurement of the lactic acid produced after 45 minutes of incubation in the presence of 10^{-5} M, 5×10^{-5} M and 10^{-4} M DNP showed that 14·4, 20·9 and 20 μmoles had been produced, respectively (Fig. 1). In the absence of DNP only 9·8 μmoles lactic acid were found. After another 50 minutes incubation in the presence of 10^{-4} and 5×10^{-5} M DNP in which hardly, if any, oxygen was taken up, 22·6 and 22·0 μmoles lactic acid were present. These data suggest that the buffer capacity of the medium (0·01 M phosphate) was too weak to permit further glycolysis and respiration *in the presence of DNP* after a critical quantity of lactic acid had accumulated.

Similar experiments were conducted in a medium with increased buffer capacity, *i.e.* 0·03 M instead of the 0·01 M phosphate buffer used in the above experiments. Fig. 2 shows that 10^{-4} and 5×10^{-5} M DNP stimulated the respiration of the ascites cells in 0·03 M phosphate buffer with glucose present over longer periods than in the 0·01 M phosphate medium (the experiments illustrated in Fig. 1 and 2 have been performed on two successive days with ascites cells from one transplant series). In the presence of 10^{-4} M DNP the respiration began to level off after 50 minutes of incubation; the same phenomenon was noted at a later time when 5×10^{-5} M DNP was present. In both cases approximately 60 μmoles lactic acid had been formed after 150 minutes at which time the rate of respiration had dropped to a minimal value.

The endogenous respiration of the cells ($Q_{O_2} = 10·5$) in the present experiment was somewhat lower than the respiration obtained in the presence of glucose *plus* 10^{-5} M DNP, which is illustrated in Fig. 2. The above results, thus, show that the Crabtree effect (drop in Q_{O_2} from 10·5 to 6·7) can be completely abolished by uncoupling of the oxidative phosphorylations of the mitochondria, and that it is even possible, under properly chosen conditions, to enhance the O_2 consumption ($Q_{O_2} = 18·3$) in the presence of glucose and DNP (5×10^{-5} M) to well over the endogenous level.

Not all cell preparations were equally sensitive to DNP and glucose; sometimes the inhibitory effect developed earlier. The latter was usually the case when ascites cells were used which had been grown in a certain batch of two- to three-months-old mice which, from later observations, appeared not to be in optimal health condition. Table I illustrates the effect of 10^{-4} M and 5×10^{-5} M DNP

on the endogenous respiration and the respiration in the presence of glucose by such cells incubated in 0·01, 0·02 and 0·03 M phosphate buffer. The general impression from these data was the same as from the earlier ones, except that in

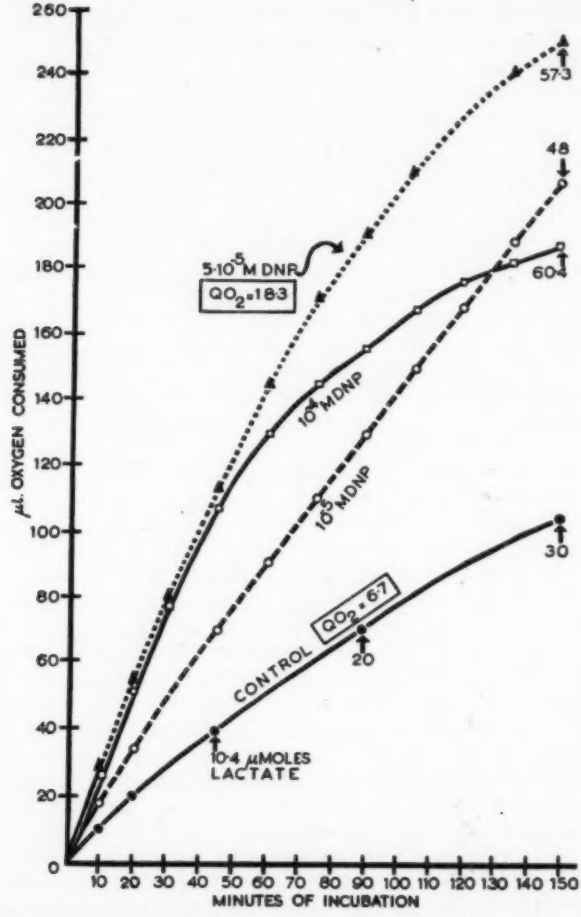

FIG. 2.—The effect of 2 : 4-dinitrophenol on the respiration of S_3A ascites carcinoma cells in presence of excess glucose.

Incubation in 1·6 ml. Krebs-Ringer phosphate (0·03 M) buffer of pH 7·4 containing 48 μmoles glucose. Figures at 90 and 150 minutes denote μmoles of lactate produced. 7·6 mg. of dry weight cells.

the 0·01 M phosphate medium the respiration became rapidly inhibited by DNP when glucose was present (*not* in its absence) and the glycolysis was only slightly enhanced.

It has been reported by Brin and McKee (1956) that addition of extra phosphate

counteracted the inhibitory effect of glucose on the respiration. In our experiments, however, such an effect has never been observed but rather the opposite was found. The endogenous respiration was not different in the 0·01 and 0·03 M phosphate buffer, and DNP also failed to show a marked effect on the endogenous respiration in the latter medium, indicating that the inorganic phosphate *per se* was not responsible for the different responses observed in the various media in the presence of DNP and glucose.

It can be seen in Fig. 1 and in Table I that from the very start of the experiments the O_2 consumption of the cells in the 0·01 and 0·02 M phosphate buffer (glucose present) was stimulated to a greater extent by 5×10^{-5} than by 10^{-4} M DNP. By contrast, in the 0·03 M phosphate buffer (Fig. 2, Table I) the initial stimulations were the same for the two DNP concentrations. Because the enhancement of the O_2 consumption is due to the uncoupling effect of DNP—the highest concentration being the most active in this respect—it follows that 10^{-4} M DNP also inhibited the respiratory processes during the whole period of incubation in the 0·01 and 0·02 M phosphate medium. Since in the latter media, but not in the 0·03 M phosphate medium, the inhibitory effect of 10^{-4} M DNP (relative to the effect of 5×10^{-5} M DNP) manifested itself momentarily and since the pH of the 0·01 and 0·02 M phosphate media did not change during the first minutes in which the inhibition was already apparent, it must be concluded that an intracellular pH change, as a result of the enhanced glycolysis, caused 10^{-4} M DNP to exert its early inhibitory effect on the respiration. It may be concluded that the inhibitory effect is dependent upon the concentration of both DNP and lactic acid, the latter being determined by the former.

TABLE I.—*Effect of DNP on Respiration and Glycolysis by S_3A Ascites Carcinoma Cells as a Function of the Buffer Strength of the Incubation Medium.*

8·5 mg./dry weight cells/flasks ; G = glucose (48 μmoles)

Phosphate buffer (M)	G	DNP (M)	μl. Oxygen consumed after (minutes) 15	30	45	60	Lactate produced after 60 minutes (μmoles)
0·01	−	..	22	45	72	95	..
	−	5×10^{-5}	30	53	80	103	..
	+	..	15	30	45	62	11·2
	+	5×10^{-5}	26	38	45	47	16·6
	+	10^{-4}	21	30	34	36	15·8
0·02	+	..	13	25	39	51	13·3
	+	5×10^{-5}	35	75	108	130	21·0
	+	10^{-4}	29	54	67	75	25·0
0·03	−	..	20	43	68	89	..
	−	5×10^{-5}	20	46	75	100	..
	+	..	12	24	36	48	14·4
	+	5×10^{-5}	35	71	105	128	25·0
	+	10^{-4}	35	70	93	108	27·2

The inhibition and final abolishment of the respiration of the cells in the presence of excess glucose and DNP was primarily due to an effect of DNP in a more acid environment and not to the pH change of the medium *per se*. Table II (Experiment 3) and Table III show that the oxygen consumption in the presence of excess glucose and in the absence of DNP was not dependent upon the pH but

that in the presence of DNP and at low pH a very marked inhibition of the O_2-uptake resulted. According to the data of Experiment 1 of Table II and similar experiments, the endogenous respiration of the cells at pH 6·5 was at its most 20 per cent lower than that at pH 7·4, while DNP stimulated at the latter but inhibited the O_2-uptake at the former pH. Since in many of our experiments the final pH after incubation with a high glucose concentration amounted to 6·7 or higher (7·4 at the start), the pronounced inhibition of the oxygen consumption by glucose (in the absence of DNP)—a Crabtree effect of 50 per cent was no exception—could not have been due to the pH change as it became manifest *extra*-cellularly. Table III provides direct evidence on this point: O_2 consumption of 53 μl. in the presence of glucose and final pH of 6·7 as against 93 μl. O_2 taken up in the absence of glucose at a pH of 6·7 during the whole period of incubation. Since in this case the endogenous respiration and the respiration in the presence of glucose are compared, and since it is known that a shift in the oxidation of endogenous to added substrates takes place, the possibility might remain that the glucose oxidation was more sensitive to the pH change than the endogenous respiration. However, the opposite was true; the oxidation of 1 mg. of [U-^{14}C] glucose by 200 mg. wet weight of cells was practically not inhibited over a pH range of 7·4 to 6·5 and the respiratory activity of cells incubated in the presence of a high glucose concentration was not (Table III) or only to a very small extent (Table II) dependent upon the pH of the medium.

TABLE II.—*Effect of DNP on the Endogenous- and Glucose-Supplemented Respiration of S_3A Ascites Carcinoma Cells as a Function of the pH.*

The last column represents the difference in O_2 uptake caused by DNP (5×10^{-5} M).

Experiment 1: 7·2 mg. dry weight of cells, no oxidizable substrate added, 0·01 M phosphate buffer;

Experiment 2: 6·4 mg. dry weight of cells, 3 μmoles glucose, 0·01 M phosphate buffer;

Experiment 3: 6·5 mg. dry weight of cells, 48 μmoles glucose, 0·03 M phosphate buffer;

Values in parentheses represent pH of the medium after 75 minutes' incubation.

Experiment	Initial pH	μl. O_2 consumed/60 minutes: DNP absent	μl. O_2 consumed/60 minutes: DNP present	Change in O_2-uptake (μl.) due to DNP
1	7·4	66	76	+10
	7·0	62	66	+4
	6·5	57	45	−12
	6·0	44	20	−24
	5·5	37	17	−20
2	7·4	57	90	+33
	7·0	50	55	+5
	6·5	47	38	−9
	6·0	40	24	−16
	5·5	37	21	−16
3	7·4	28 (6·7)	70 (6·6)	+42
	7·0	33 (6·4)	48 (6·2)	+15
	6·5	29 (6·0)	27 (5·2)	−2
	6·0	25 (4·5)	14 (4·2)	−11
	5·5	23 (4·2)	5 (4·2)	−18

It is of interest that an *intra*cellular pH change as a result of glucose catabolism by the ascites cells has been considered (Bloch-Frankenthal and Weinhouse, 1957) to be responsible for or to contribute to the inhibition of the oxidative processes. However, it should be noted that the intracellular pH change which caused DNP to exert its early inhibitory effect on the uncoupled oxidation, could be counteracted by increasing the buffer strength of the medium, whereas the inhibition of the oxidation in the presence of glucose alone manifested itself momentarily, regardless of the buffer capacity (0·01, 0·02 and 0·03 M phosphate) of the suspension fluid. Moreover, the fact that the O_2 consumption can be enhanced substantially by adding DNP whereas at the same time the lactic acid formation from glucose is doubled, discourages the interpretation that an intracellular pH change *per se* might be responsible for an inhibition of the hydrogen and electron transport in the respiratory chain. In the latter argument the situation in the presence of DNP and glucose is taken as representative of that in the presence of glucose alone. However, since DNP abolishes the mitochondrial phosphorylation, the possibility might remain that glucose catabolism in the absence of DNP causes an intracellular pH change which would inhibit the rate of the phosphorylations associated with the hydrogen and electron transport in the mitochondrial respiratory chain and thus retard the O_2-uptake. To the best of our knowledge, however, the available evidence does not favour this supposition.*

TABLE III.—*The Effect of the pH on the Respiration of S_3A Ascites Carcinoma Cells Incubated in the Absence and Presence of Glucose*

Glucose (48 μmoles) added as indicated. Values in parentheses represent the pH after incubation ; (—) no change. Incubation during 75 minutes at 37° C. in Krebs-Ringer phosphate (0·03 M) buffer ; 8·5 mg. of dry weight cells.

Initial pH	μl. O_2 consumed	
	Glucose present	Glucose absent
7·4	53 (6·7)	110 (—)
7·0	60 (6·2)	98 (—)
6·5	55 (5·5)	89 (—)
6·0	65 (4·4)	70 (—)
5·5	58 (4·2)	55 (—)

Effect of 2:4-dinitrophenol on the respiration in the presence of a small amount of glucose

The overall O_2-uptake of the ascites cells during 60 minutes was not affected when incubation was carried out in the presence of a small amount of glucose (1–2 μmoles, 0·01 M phosphate buffer). With prolonged incubations (from 60–180 minutes) the rate of the endogenous respiration dropped somewhat but remained linear in the presence of 1–2 μmoles of glucose. DNP (5×10^{-6} M) stimulated the latter O_2-uptake over the whole 3-hour period. In experiments conducted

* Another possibility must be kept in mind, namely that the intracellular lactic acid does not readily equilibrate with the medium and that DNP, besides enhancing lactic acid production, might change the permeability of the cells and thus greatly accelerate the exit of acid. Our recent experiments show that lactate does indeed accumulate to some extent in the cells but that in the presence of DNP even more lactate is present intracellularly.

during 60 minutes with 1 μmole of glucose and DNP, the O_2 consumption was raised to the same level, regardless of the fact whether glucose and or DNP (5×10^{-5} M) were present from the start of the experiment or added after 15 minutes (Table IV). In those cases in which DNP was added after 15 minutes (Table IV, Experiments d and e) the O_2-uptake showed an immediate and sharp rise. The stimulatory effect of DNP on the endogenous respiration was more pronounced when the uncoupling agent was added 15 minutes after closing the stopcocks of the respirometers, than when it had been present from the beginning (Table IV, Experiments g and h); a 50 per cent stimulation of the endogenous respiration has thus been obtained.

TABLE IV.—*Effect of DNP, added at Various Times, on the Respiration of S_3A Ascites Carcinoma Cells in the Absence and Presence of 1 μmole Glucose*

7·0 mg. dry weight of cells suspended in 0·01 M phosphate buffer. Values in parentheses indicate the time in minutes at which glucose or DNP added to the main compartment of the respirometers.

Experiment	DNP (5×10^{-5} M)	Glucose (1 μmole)	μl. O_2 taken up in 60 minutes
a	−	+ (0)	65
b	+ (0)	+ (0)	100
c	−	+ (15)	63
d	+ (15)	+ (15)	107
e	+ (15)	+ (0)	107
f	−	−	66
g	+ (0)	−	78
h	+ (15)	−	98

These results indicate that a more pronounced stimulation of the respiration, in the absence or presence of low amounts of glucose, can be effected by DNP when the cells are allowed to mobilize (endogenous) substrate or produce a certain amount of ATP prior to the addition of DNP. In this connection the following results are also of interest. In a few, out of numerous experiments, DNP (5×10^{-5} M, present from the start of the experiment) caused a severe inhibition of the endogenous respiration (Fig. 3) which was completely counteracted by the simultaneous presence of 1 μmole glucose; however, when DNP was added after 15 minutes the O_2-uptake resulting from the endogenous respiration was not markedly affected. In a similar experiment ran in the presence of 1 μmole glucose, 5×10^{-5} M DNP (present from the start) stimulated the O_2 consumption for 30 per cent but 10^{-4} M DNP caused an inhibition of more than 50 per cent; when DNP was added in the latter concentration after 15 minutes, a slight stimulation of the respiration was observed instead. The results suggest that the inhibition by DNP is due to a lack of oxidizable substrate (*e.g.* acyl-coenzyme A, which is dependent upon ATP for its formation, in the case of the endogenous respiration) and/or a certain level of ATP which is necessary to guarantee the intactness of the oxidative systems (*e.g.*, coenzyme-binding).*

It must be noted, finally, that glycolysis and oxidation of glucose by ascites cells proceed at a maximal or near-maximal rate at very low concentrations of

* The oxidation of glutamate by isolated mouse liver mitochondria is, in our hands, inhibited by DNP at pH 7·0 (in contrast to the enhancement noted at pH 7·4), the inhibition being counteracted by the addition of diphosphopyridine nucleotide.

glucose, which do not give rise to a Crabtree effect. Since the theory which links the Crabtree effect with phosphorylations presupposes that the inhibition of respiration by glucose is directly associated with the intensity of glycolysis, the former observations may invalidate the theory (Bloch-Frankenthal and Weinhouse, 1957). We have, however, been able in the present experiments conducted with the S_3A ascites carcinoma cells, to observe an inhibition of the respiration in the presence of 1 or 2 μmoles of glucose, added at zero time or

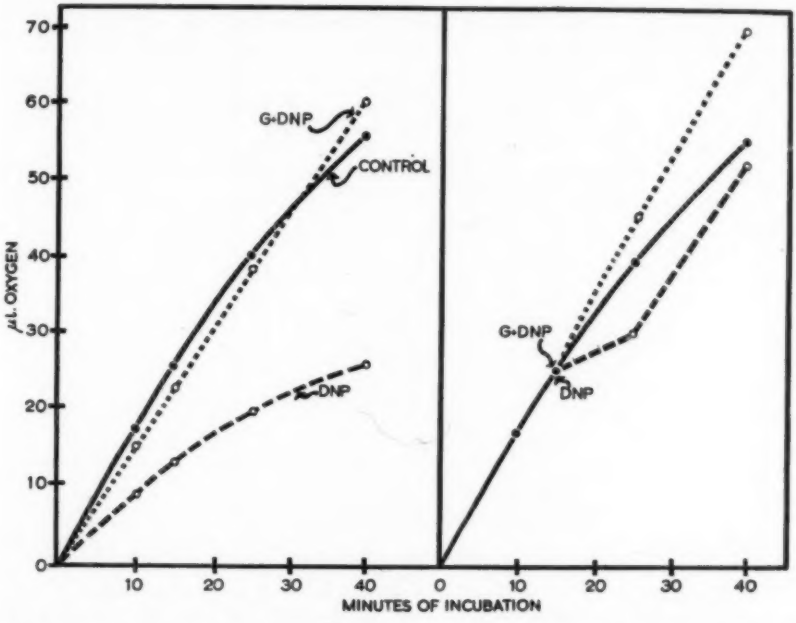

FIG. 3.—The effect of 2 : 4-dinitrophenol on the respiration of S_3A ascites carcinoma cells in the absence and presence of 1 μmole glucose—(see text).

Control represents endogenous respiration. 5×10^{-5} M DNP added as indicated—left side: DNP and glucose (G) + DNP present from the start; right side: added after 15 minutes. 7·8 mg. dry weight of cells were present. The experiment was continued for 120 minutes.

after 15 minutes (Fig. 4), which lasted for a short period of time only. A definite lag time was observed in the O_2-uptake which lasted for 10 minutes after the addition of glucose, followed by a recovery in the rate of the O_2-uptake so that the overall O_2-uptake of the cells in one hour period was not affected. When DNP (5×10^{-5} M) was added together with the glucose no lag in the respiration occurred. These results are in accord with the view that the mitochondrial oxidations are temporarily hampered by the active glycolysis. When the latter process diminished and came to a stop or when the mitochondrial phosphorylations were abolished, the oxidations regained, or even surpassed, the initial rate.

Effect of 2:4-dinitrophenol on respiration in the presence of pyruvate and succinate

The effect of DNP on the oxidation of pyruvate by the S_3A ascites cells was investigated over a period of 180 minutes. Fig. 5 illustrates the results obtained during the first 60 minutes of a typical experiment carried out in 0·01 M Krebs-Ringer phosphate buffer. Pyruvate (16 μmoles) caused no, or very slight, stimulation of the respiration of the cells above the endogenous level during the first hour of incubation. After longer incubation periods the endogenous respiration

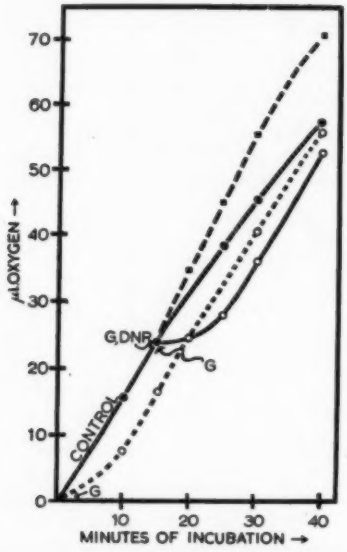

FIG. 4.—The effect of 1 μmole glucose on the respiration of S_3A ascites carcinoma cells. Control represents endogenous respiration; glucose (G) added at zero time or after 15 minutes; G + DNP added after 15 minutes. 7·7 mg. dry weight of cells were present.

diminished (slightly), but in the presence of pyruvate the respiration remained essentially linear. After 180 minutes only 3 μmoles of lactate were produced from pyruvate, and 2·8 μmoles from pyruvate incubated in the presence of 10^{-4} M DNP. The control flasks (no pyruvate added) incubated with or without DNP, contained only trace amounts of lactic acid. 10^{-4} M now caused a greater stimulation than 5×10^{-5} M DNP over the whole 3 hour period of incubation with pyruvate. The O_2-uptake was enhanced nearly 2-fold by 10^{-4} M DNP ($Q_{O_2} = 20$) while the endogenous respiration ($Q_{O_2} = 11$) was not affected. Since 5×10^{-5} M DNP showed some stimulatory effect on the endogenous respiration it follows that the two DNP concentrations had a different effect on the endogenous oxidation and that of pyruvate.

The effect of DNP on the respiration of the ascites cells in the presence of succinate was similar to that observed in the presence of pyruvate (Table V).

TABLE V.—*Effect of DNP on the Respiration of S_3A Ascites Carcinoma Cells Incubated in the Presence of Pyruvate and Succinate*

Incubation during 80 minutes with 7·3 mg. of wet weight cells ($Q_{O_2} = 10$) in Krebs-Ringer phosphate (0·01 M) buffer, 20 μmoles of substrate added.

Substrate	μl. O_2 consumed		
	DNP absent	DNP 10^{-4} M	DNP 5×10^{-5} M
.. . . .	97	91 .	115
Pyruvate . . .	90	165 .	160
Succinate . . .	121	185 .	178

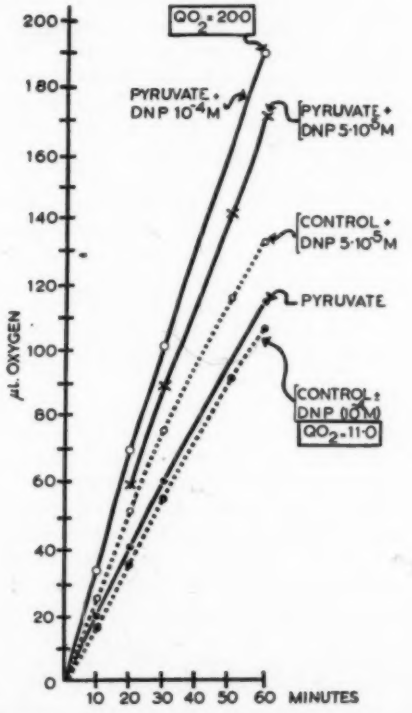

FIG. 5.—The effect of 2 : 4-dinitrophenol on the respiration of S_3A ascites carcinoma cells in the presence of pyruvate.
Pyruvate 16 μmoles; 9·5 mg. dry weight of cells.

Effect of citric acid cycle intermediates on respiration and glycolysis

Addition of α-ketoglutarate, malate or succinate (24 μmoles each) to the S_3A ascites cells led to an enhancement of the O_2 consumption. The enhancement was always most pronounced in the presence of succinate. On addition of glucose the Crabtree effect manifested itself, though the magnitude of the effect was

usually (somewhat) smaller than that observed in the absence of the citric acid cycle intermediates. However, the oxygen consumption in the presence of excess glucose plus citric acid cycle intermediate was higher than that taking place in the presence of glucose alone. This counteraction of the Crabtree effect must, according to the standard interpretation, be due to the fact that the mitochondrial phosphorylations compete more favourably with the glycolytic ones when the concentration of the citric acid cycle intermediates is enhanced, and thus allow a higher oxygen uptake. If this conclusion is correct, the increase in the O_2 consumption, following the addition of a certain citric acid cycle substrate, should be accompanied by a decrease in the aerobic glycolysis and the latter should be proportional to the P:O ratio of the particular oxidation as known from studies with isolated mitochondria. Such studies have shown that the oxidation of various substrates differ markedly in their dependence upon the amount of ADP and P_i taken up per atom oxygen consumed; liver mitochondria show P:O ratios of respectively 2, 3, and 4 in the presence of succinate, malate and α-ketoglutarate, respectively. The present results, some of which are illustrated in Table VI, showed that the increase in the O_2 consumption following the addition of each citric acid cycle substrate to the ascites tumour cells was accompanied by a decrease in the aerobic lactate production and that the ratio of the latter relative to the former (Table VI, Δ lactate : Δ oxygen ratio) was smaller for succinate than for malate, whereas that of α-ketoglutarate was higher than that of malate. This sequence shows an exact parallel with that of the P :O ratios of isolated liver mitochondria. It is, therefore, concluded that the present results furnish strong evidence for the fact that the mitochondrial oxidations of the three citric acid cycle intermediates by intact ascites tumour cells require different amounts of ADP and P_i, in accordance with what is known from studies with isolated mitochondria.

The inhibition of glycolysis in the presence of a citric acid cycle intermediate has been further characterized :

(i) The inhibition was found to be dependent upon the presence of oxygen, that is upon respiration. Under anaerobic conditions (both in phosphate- and bicarbonate buffer) succinate and malate did not decrease the lactate production but rather increased it somewhat. In the presence of α-ketoglutaric acid, however, a small drop (20–25 per cent) in anaerobic glycolysis was observed in most experiments. This might have been due to the substrate phosphorylation accompanying the anaerobic dehydrogenation of α-ketoglutarate

$$(\rightarrow \text{succinyl coenzyme A} \xrightarrow{\text{ADP, } P_i} \text{succinate}),$$

since the phosphorylations in the respiratory chain do not take place under anaerobic conditions, the reduced coenzymes being used to some extent in reductive syntheses. Coupled oxido-reduction reactions do occur under aerobic conditions since it has been shown that the respiratory quotient of ascites cells in the presence of excess glucose may reach a value of 1·25. Accordingly, citric acid cycle intermediates may be metabolized to some extent under anaerobic conditions. The present results may furnish circumstantial evidence for a competition between the substrate level phosphorylations of glycolysis and the only one of this kind in the citric acid cycle. This competition is apt to occur since it can be shown that even under anaerobic conditions the rate of glycolysis of the intact S_3A

mammary carcinoma cells is limited by the ATP-turnover (Emmelot and Bos, 1959).

TABLE VI.—*Effect of Citric Acid Cycle Intermediates on the Aerobic Glycolysis and Respiration of S_3A Ascites Carcinoma Cells*

Glucose (24 μmoles) present in each flask and citric acid cycle intermediate (24 μmoles) added as indicated. Incubated during 60 minutes in Krebs-Ringer phosphate buffer, dry weight of cells 6–8 mg. Values in parentheses represent the O_2 uptake in the absence of glucose. In the last experiment the effect of DNP (10^{-4} M) is illustrated : incubation during 40 minutes with 10 μmoles α-ketoglutarate.

Medium	Substrate	Oxygen consumption (μatoms)	Lactate production (μmoles)	$\frac{\Delta \text{ Lactate}}{\Delta \text{ Oxygen}}$
0·01 M phosphate	—	5·4 (10·1)	11·8	—
+ 0·01 M Tris	Succinate	8·8 (13·3)	10·0	0·5†
	Malate	7·7 (11·7)	9·2	1·1
	α-Ketoglutarate	7·5 (11·2)	5·7	2·9
0·03 M phosphate	—	5·9 (9·9)	15·0	—
	Succinate	7·3 (12·4)	14·1	0·6
	Malate	7·1 (10·3)	12·8	2·6[5]
	α-Ketoglutarate	7·2 (10·2)	9·4	4·3
,, ,, ,,	—	4·3	11·7	—
	Succinate	5·8	10·0	1·1
	Malate	5·5	8·3	2·8
	α-Ketoglutarate	5·5	5·7	5·0
0·01 M phosphate	—	3·0	7·8	—
+ 0·01 M Tris	α-Keotglutarate	3·9	3·9	—
	DNP	5·5	13·8	—
	α-Ketoglutarate + DNP	6·6	13·3	—

† Calculated as $(11·8 - 10·0)/(8·8 - 5·4)$.

(ii) The glycolytic inhibition under aerobic conditions was independent of the concentration of α-ketoglutarate in the range of 5–24 μmoles.

(iii) The inhibition of the aerobic glycolysis by α-ketoglutarate and malate was completely abolished by the presence of 10^{-4} M DNP (Table VI, same result obtained in bicarbonate buffer); only in some experiments with α-ketoglutarate a very slight inhibition remained. In fact, with α-ketoglutarate, glucose and DNP a small inhibition of the aerobic glycolysis might have been expected, comparable in magnitude to that observed under anaerobic conditions as a result of α-ketoglutarate addition. It will, however, be shown in the following paper (Emmelot and Bos, 1959) that DNP not only abolished the oxidative phosphorylation in the respiratory chain but actually activated the mitochondrial ATPases of the ascites cells.

(iv) The inhibition of the aerobic glycolysis by α-ketoglutarate was only observed when mitochondria were present; in 20,000 $\times$ **g** supernatants of disrupted ascites tumour cells no inhibition occurred, whereas after addition of liver mitochondria to the latter system a large inhibition of glycolysis resulted from the extra addition of α-ketoglutarate.

(v) The marked inhibition of the aerobic glycolysis by α-ketoglutarate in broken ascites tumour cell preparations and in combinations of 20,000 $\times$ **g**

tumour cell supernatants and liver mitochondria, could be abolished by adding ADP or AMP.

Detailed results of these experiments will be reported separately.

Comment.—The latter experiments prove that mitochondrial oxidations and extra-mitochondrial glycolysis are competitive reactions; they also strongly suggest that the competition occurs between the phosphorylations which accompany the two processes in intact ascites tumour cells, or at least that mitochondrial oxidative phosphorylations are inhibitory to glycolysis. The marked enhancement by DNP of the oxidation of pyruvate and succinate (two substrates which do not give rise to the Crabtree effect) also clearly shows that in the absence of glucose the rate of functioning of the citric acid cycle is dependent upon the accompanying phosphorylations in the respiratory chain. Since the respiration of the glucose-inhibited cells ($\ll$ endogenous respiration) and the respiration in the presence of the citric acid cycle intermediates pyruvate and succinate ($\geqslant$ endogenous respiration, glucose absent), could be raised to the same high level by abolishing the mitochondrial phosphorylations, it follows that the Crabtree effect (inhibition of the endogenous respiration by glycolysis) operates at the level of the mitochondrial phosphorylations. The present results are compatible with the view that the latter phosphorylations are inhibited by the glycolytic phosphorylations which leave only a limited amount of ADP and P_i available to the mitochondria. The distribution of ADP and P_i over the cell-sap and the mitochondria of ascites cells incubated in the presence of glucose may apparently be altered in favour of the mitochondria by adding a citric acid cycle intermediate, since oxidation then increases and the aerobic glycolysis decreases. In the latter case, a greater part of the total ATP is generated in the mitochondria as compared with the situation in the presence of glucose alone. The possibility that this mitochondrial ATP may not readily equilibrate with the cell-sap and thus retard the ATP-dependent phosphorylations of glucose—a suggestion made to explain the lowered glucose uptake accompanying the Pasteur effect—may tend to favour the decrease in the aerobic glycolysis.

From the present results two general conclusions regarding mitochondrial oxidation and phosphorylation in ascites tumour cells may be drawn.

(i) Oxidative phosphorylation does occur in the respiratory chain of mitochondria of intact ascites tumour cells. Studies with isolated mitochondria from such cells are in line with this concept since we have obtained P:O ratios of 2·3 with glutamate in the presence of glucose-hexokinase. The latter result indicates that the enzymes of all three phosphorylating steps are present. Moreover, it has been shown (Emmelot and van Vals, 1957; Emmelot and Bos, 1959) that the energy (ATP) derived from the endogenous oxidations of the ascites tumour cells is equivalent to that derived from aerobic glycolysis plus glucose oxidation, or from anaerobic glycolysis, in sustaining amino acid incorporation into the proteins of the ascites cells. A similar conclusion has recently also been reached by Quastel and Bickis (1959).

(ii) Since in the presence of DNP the respiration of ascites tumour cells may be raised to a Q_{O_2} value of 20 and higher (compare also Woods, 1956), and since the active glycolysis appears to inhibit the respiration, there is no reason to assume that in the ascites tumour cell the high glycolysis compensates for an "irreversibly injured" respiration, as suggested in Warburg's (1956) theory on the origin of cancer cells.

SUMMARY

The effect of DNP on the endogenous and the glucose-supplemented respiration of ascites tumour cells has been studied in some detail. It is concluded that DNP may both stimulate and inhibit the oxidations at the same time, the magnitude of each effect being a function of the DNP concentration, the "quality" of the cells and, if glucose is present, of the pH. The endogenous respiration is in general little affected by DNP; at 5×10^{-5} M the stimulation predominates but at 10^{-4} M DNP the inhibition may become evident. The inhibitory aspect of DNP action is counteracted by a short-time pre-incubation of the cells in the absence of the uncoupling agent. In exceptional cases, DNP causes a severe inhibition of the respiration that can be prevented either by preliminary incubation of the cells or by the addition of a very small amount of glucose. When incubation is carried out in the presence of a very low glucose concentration, the stimulatory effect of DNP predominates over the inhibitory effect, while the O_2-uptake reaches a higher level than that of cells incubated with DNP in the absence of glucose. In the presence of excess glucose DNP markedly enhances the oxidations and completely counteracts the Crabtree effect. Upon lowering the pH of the medium the endogenous oxidation decreases, but the respiration in the presence of excess glucose changes hardly, if at all; however, in both instances the inhibitory effect of DNP increases with decreasing pH. Since DNP stimulates the lactic acid production of the ascites cells markedly in the presence of excess glucose, the intracellular pH may drop during the incubation to such an extent that an inhibition, and finally a complete suppression, of the respiration by DNP results; some cell preparations are more sensitive in this respect than others. The latter inhibition is a function of the pH and the DNP concentration but it can be completely counteracted by increasing the buffer strength of the medium. Since the respiration of the ascites tumour cells in the presence of excess glucose or citric acid cycle intermediates may be enhanced by DNP to Q_{O_2} values of 20 or higher. Warburg's concept of an "irreversibly-injured" respiration of cancer cells is discounted.

These and other data have yielded results which support the view that the competition between the glycolytic and oxidative reactions of the ascites tumour cells operates at the level of the phosphorylations accompanying the two processes. This competition leads to a marked inhibition of the glycolysis by respiration (Pasteur effect), of the endogenous respiration by glycolysis (Crabtree effect), of the aerobic glycolysis when the respiration in the presence of excess glucose is enhanced by adding citric acid cycle intermediates, and also to a small inhibition of the anaerobic glycolysis when the cells are allowed to metabolize glucose and α-ketoglutarate simultaneously.

REFERENCES

BARKER, S. B.—(1957) 'Methods in Enzymology.' Ed. by S. P. Colowick and N. O. Kaplan; New York (Academic Press) vol. 3, 241.
BLOCH-FRANKENTHAL, L. and WEINHOUSE, S.—(1957) *Cancer Res.*, **17**, 1082.
BRIN, M. AND MCKEE, R. W.—(1956) *Ibid.*, **16**, 364.
CHANCE, B. AND HESS, B.—(1956) *Ann. N.Y. Acad. Sci.*, **63**, 1008.
CRABTREE, H. G.—(1929) *Biochem. J.*, **23**, 536.

EMMELOT, P. AND BOS, C. J.—(1957) *Enzymologia*, **18**, 149, 179.—(1959) *Brit. J. Cancer*, **13**, 537.
Idem AND NOUT, S. J.—(1959) *Ibid.*, **13**, 513.
Idem AND VAN VALS, G. H.—(1957) *Ibid.*, **11**, 620.
IBSEN, K. H., COE, E. L. AND MCKEE, R. W.—(1958) *Biochim. biophys. Acta*, **30**, 384.
JOHNSON, M. S.—(1941) *Science*, **94**, 200.
KUN, E., TALALAY, P. AND WILLIAMS-ASHMAN, H. G.—(1951) *Cancer Res.*, **11**, 855.
KVAMME, E.—(1958) *Acta physiol. scand.*, **42**, 204, 219, 231, 239.
LOOMIS, W. F. AND LIPMANN, F.—(1948) *J. biol. Chem.*, **173**, 807.
LYNEN, F.—(1941) *Liebigs Ann.*, **546**, 120.—(1956) *Int. Congr. Biochem.*, Brussels, 1955, **3**, 294.—(1958) in ' Neuere Ergebnisse Chemie u. Stoffwechsel der Kohlenhydrate.' Berlin (Springer Verlag), p. 155.
MEDES, G. AND WEINHOUSE, S.—(1958) *Cancer Res.*, **18**, 352.
POTTER, V. R. AND LYLE, G. G.—(1951) *Ibid.*, **11**, 355.
QUASTEL, J. H. AND BICKIS, I. J.—(1959) *Nature, Lond.*, **183**, 281.
RACKER, E.—(1956) *Ann. N.Y. Acad. Sci.*, **63**, 1017.
SEELICH, F., WEIGERT, W. AND LETNANSKY, K.—(1956) *Z. Krebsforsch.*, **61**, 368.
WARBURG, O.—(1956) *Science*, **123**, 309.
WOODS, M.—(1956) *J. nat. Cancer Inst.*, **17**, 615.

THE METABOLISM OF NEOPLASTIC TISSUES: THE EFFECT OF 2:4-DINITROPHENOL ON GLYCOLYSIS AND ATP-DEPHOSPHORYLATION OF ASCITES TUMOUR CELLS AND HOMOGENATES*†

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RESPIRATION and glycolysis are both processes which require inorganic phosphate and adenine nucleotides. The inhibition of glycolysis by respiration (Pasteur effect) in both normal and neoplastic tissue, and the reverse situation—the inhibition of respiration by glycolysis (" reversed Pasteur effect " or Crabtree effect)—in neoplastic tissue and especially in ascites tumour cells, may be explained on the basis of a competition between the two processes at the level of the accompanying phosphorylations (Lynen, 1956, 1958; Racker, 1956; Emmelot and Bos, 1959).

In the preceding paper the effect of DNP on the respiration of ascites tumour cells was studied. The present paper deals with an investigation of the effect of DNP on the glycolysis of these cells. The anaerobic glycolysis has received particular attention as a result of the finding that DNP may stimulate this process to a very marked extent (Emmelot and Bos, 1958*a*). It is known that DNP induces ATPase activity in isolated mitochondria incubated in the absence of oxidizable substrate, which splits added ATP into ADP and P_i. According to our results the same phenomenon occurs also in intact ascites tumour cells.

MATERIALS AND METHODS

The various tumours used in the present investigation have been described previously (Emmelot and Bos, 1955; Emmelot *et al.*, 1959).

Ascites tumour cells corresponding with 5–9 mg. dry weight of cells in the various experiments were suspended in 1·6 ml. Krebs-Ringer bicarbonate buffer containing 48 μmoles glucose and incubated at 37° C. Phosphate buffer has been used only in the experiments listed in Table I. In a number of experiments ascites fluid fortified with bicarbonate was used as suspending medium. No differences in results were obtained with those carried out in ordinary bicarbonate buffer and reported in this paper.

Cell suspensions were prepared from a solid lympho-sarcoma by a modification of the procedure of Freund and Kaminer (1910). The tumour tissue, moistened with an acid buffer (1 per cent KH_2PO_4 plus 0·6 per cent NaCl; pH 5·4) in a

* The main results of this investigation were communicated at the 7th International Cancer Congress, London, July, 1958.

† Abbreviations are used as follows: P_i for inorganic phosphate, AMP, ADP, and ATP for adenine mono-, di- and triphosphates, DNP for 2:4-dinitrophenol.

Petri dish placed in ice, was cut to very small pieces with a pair of scissors. The *brei* was diluted with cooled buffer and directly filtered through 8 layers of aseptic gauze. Centrifugation was carried out at very low speed for 2 minutes, the sediment being washed once with and suspended in physiological saline. Aliquots of the latter suspension were used immediately. The whole procedure was carried out as quickly as possible at a temperature of 0° C. The final suspension was checked by microscopy and found to contain only free cells ; no cell clumps were observed. The lymphosarcoma cells thus obtained showed a vigorous glycolysis and a marked Pasteur effect indicating that their enzymic machinery was not damaged to any marked extent.

The S_3A mammary ascites carcinoma cells were ruptured by suspension in ice-cold bidistilled water for 10 minutes, followed by homogenisation during 30 seconds in an all-glass apparatus according to Potter-Elvejhem, using a rather tight-fitting pestle. The final water homogenates were checked by phase-contrast microscopy ; intact cells were scarce. Aliquots of the homogenates were incubated in the medium according to LePage (1948) with the omission of fructose diphosphate and in the absence or presence of potassium fluoride (see text).

Phosphate was measured according to Fiske and Subbarow (1929) and lactic acid according to Barker (1957).

Glucose was determined with glucose oxidase using the reagents of, and according to the directions provided by, Boehringer u.S. (Mannheim, Germany).

TABLE I.—*Effect of* 2: 4-*Dinitrophenol on Glycolysis and Glucose Consumption by* S_3A *Ascites Carcinoma Cells suspended in Krebs-Ringer Phosphate Buffer.* Incubation During 35 Minutes with 8 mg. of Dry Weight Cells; 10^{-4} M DNP

Condition	DNP	Lactate production (micromoles)	Glucose consumption (micromoles)	
			Found	Calculated
Anaerobic (100% N_2)	−	7·0	3·4	3·5
	+	12·0	5·9	6·0
Aerobic (air)	−	5·4	2·9	2·7
	+	12·5	6·7	6·25

RESULTS AND DISCUSSION

Effect of 2: 4-*dinitrophenol on the aerobic and anaerobic glycolysis of the* S_3A *ascites carcinoma*

Freshly harvested S_3A ascites carcinoma cells contained few erythrocytes and one washing with physiological saline sufficed to obtain a colourless pellet. It is shown in Table I that addition of 10^{-4} M DNP to ascites tumour cells suspended in 0·01 M phosphate buffer of pH 7·4 at 37° C. abolished the Pasteur effect and stimulated the aerobic glycolysis well beyond the anaerobic level reached in the absence of DNP. The glucose consumption was accounted for by the lactate production.

The same experiments were also carried out in Krebs-Ringer bicarbonate buffer since the latter medium afforded a better protection against accumulating lactic acid than the phosphate medium, in which glycolysis was not optimal (Emmelot and Bos, 1959). In a number of experiments it was found that the aerobic glycolysis of the ascites cells in the bicarbonate medium was equal to

the anaerobic glycolysis (Emmelot and Bos, 1958*a*). This situation has been encountered only with the S_3A ascites carcinoma suspended in the bicarbonate medium; it has never been observed when these cells were suspended in the phosphate medium. In contrast, the anaerobic glycolysis was always higher than the aerobic glycolysis in two other ascites tumours (Ehrlich carcinoma and T 86157 lymphosarcoma), regardless of the nature of the suspending medium. Since the conditions under which the cells were allowed to glycolyse anaerobically were indeed strictly anaerobic (compare below), the absence of the Pasteur effect in a number of experiments with the S_3A ascites tumour cells cannot easily be explained. Two of these experiments are listed in Table II (Experiments 1 and 2). However, in many other experiments with the S_3A ascites tumour cells the Pasteur effect was observed (Table II; Experiments 3–6 are typical for the latter results). In all experiments conducted in the bicarbonate medium, DNP markedly stimulated both the aerobic and anaerobic glycolysis; the stimulation was higher than that observed in the phosphate medium—in the latter medium an average stimulation of 40 per cent of the anaerobic glycolysis by DNP was noted.

TABLE II.—*Effect of* 2: 4-*Dinitrophenol on the Glycolysis of* S_3A *Ascites Carcinoma Cells Suspended in Krebs-Ringer Bicarbonate Buffer*

Experiment (No.)	Dry weight of cells (mg.)	Incubation (minutes)	Anaerobic (95% N_2, 5% CO_2) (micromoles of lactate produced)		Aerobic (95% O_2, 5% CO_2) (micromoles of lactate produced)	
			− DNP	+ DNP	− DNP	+ DNP
1	8·0	20	6·3	14·5	6·1	15·1
2	4·5	40	5·8	22·7	5·5	20·0
3	7·5	20	9·0	16·5	5·8	16·5
4	5·4	45	11·9	20·3	7·3	21·1
5	6·0	60	15·6	27·8	8·8	29·0
6	6·2	40	7·3	13·1	3·2	14·0

An effect of DNP on the aerobic glycolysis of the ascites cells may be expected in view of the uncoupling of the oxidative phosphorylations by this agent. However, the magnitude of this effect and the finding that the anaerobic glycolysis was also markedly enhanced was quite unexpected and led us to a closer investigation of the effect of DNP on the glycolysis of normal and neoplastic tissues.

Effect of 2: 4-*dinitrophenol on the aerobic and anaerobic glycolysis of normal tumour cells*

DNP (10^{-4} M) was found to abolish the Pasteur effect in all normal and tumour cells listed in Table III, but the pronounced enhancing effect of the uncoupling agent on the anaerobic glycolysis appeared to be specific for free ascites tumour cells. The anaerobic glycolysis of the Ehrlich ascites carcinoma (Table III) responded in a similar manner towards DNP as the corresponding process in the S_3A ascites carcinoma. By contrast, DNP had no effect on the anaerobic glycolysis of slices of solid tumours or of two *normal* types of free cells, *i.e.*, bone marrow cells and lymphoblasts (strain 3′) derived from the lymphosarcoma T 86157 through subculture *in vitro* by Dr. W. C. de Bruyn of this Institute. The strain 3′, in contrast to another strain (6 A) derived from the same tumour, has lost the capacity to induce a tumour on reintroduction in the mouse. It was of interest that DNP did not increase the anaerobic glycolysis of strain 6 A cells either.

The anaerobic glycolysis of slices of the parent tumour T 86157, maintained in the solid form, was also not affected by DNP, but a positive effect was observed with cells of the same tumour grown in the ascites form. A suspension of free cells prepared from the solid tumour T 86157 showed a high glycolytic rate and DNP stimulated the anaerobic glycolysis provided that preferably less than 7 mg. of dry weight of the cells were incubated with the uncoupling agent in a final volume of 1·6 ml. bicarbonate buffer.

TABLE III.—*Effect of* 2 : 4-*Dinitrophenol on the Aerobic and Anaerobic Glycolysis of Normal and Neoplastic Tissues* ; 10^{-4} M *DNP*

Tissue	Dry weight (mg.)	Incubation period (minutes)	Lactate production (micromoles)			
			Anaerobic		Aerobic	
			DNP absent	DNP present	DNP absent	DNP present
Ehrlich ascites carcinoma	6·8	20	5·6	13·0	4·2	12·5
	5·2	40	6·5	15·0	4·2	15·5
T 86157 ascites lymphosarcoma	6·0	35	7·8	18·8	4·6	20·8
	3·1	35	4·2	13·5	3·0	13·3
T 86157 solid lymphosarcoma (sliced)	13·0	60	14·9	15·6	6·7	14·3
	6·5	60	8·3	8·9	3·6	7·9
T 86157 solid lymphosarcoma (cell prepn.)	12·9	60	20·9	22·3	11·0	20·2
	7·4	60	9·0	11·1	4·4	11·2
	4·2	60	4·3	7·2	2·9	7·7
	4·1	60	3·6	5·1	—	—
	3·4	60	3·5	6·0	3·0	5·8
Strain 3′ non-malignant lymphoblasts from T 86157	7·8	60	9·2	9·0	6·2	11·9
	8·1	60	10·0	10·8	6·2	13·0
Strain 6 A malignant lymphoblasts from T 86157	5·2	60	8·0	9·4	3·3	9·7
	2·8	100	4·7	4·7	1·2	4·9
Bone marrow cells (rabbit)	8·3	60	5·5	6·5	2·2	7·2
Solid tumours (slices)—						
UV 256 mouse sarcoma	15·3	60	8·9	9·8	4·1	8·3
T 28012 mouse hepatoma	15·1	60	7·1	7·7	2·7	5·7
Primary rat hepatoma (hepatocellular type)	15·9	60	10·2	9·1	5·1	8·5
BY 252 transplanted rat hepatoma (hepatocellular type)	15·0	60	16·0	14·3	8·5	19·0
	16·9	60	20·2	22·8	9·2	18·9

It is seen in Table II and Table III that 10^{-4} M DNP increased the anaerobic glycolysis from 1·5 to 3-fold in the various expriments with the three ascites tumours. A nearly two-fold stimulation was observed in most cases with the S_3A ascites tumour (Table II, Experiments 3–5). The marked stimulation of the anaerobic lactate production of the ascites tumour cells observed in the present experiments by DNP is without precedent* (compare Simon, 1953). In the case

* In a recent discussion at the Ciba Foundation Symposium on the Regulation of Cell Metabolism (Churchill Ltd., London, 1959) Greville (p. 12 and 255) mentioned another instance in which DNP vastly stimulates anaerobic glycolysis (intact frog muscle).

of yeast, for instance, only a very small effect has been noted (Stickland, 1956). However, in the course of preparing the present paper a publication by Clowes and Keltch (1954) came to our attention in which the anaerobic lactate production of sarcoma 180 ascites cells was reported to be stimulated 1·7-fold by dinitrocresol.

In an attempt to account for our findings the following hypothesis was proposed (Emmelot and Bos, 1958*a*). The glycolytic potential of the ascites cells might be so high that even under anaerobic conditions when most of the P_i and ADP of the cells is available to the cytoplasmic glycolysis, the actual rate of the latter process remains below that attainable if the steady-state concentrations of P_i and ADP were to be raised. In other words, the anaerobic glycolysis was governed by the (slower) rate of ATP-dephosphorylation. Now, DNP is known not only to uncouple the oxidative phosphorylations in the mitochondria but also to induce an active ATP-splitting enzyme which converts ATP into ADP and P_i (DNP activation of the latent ATPase). Activation of the ascites tumour mitochondrial ATPases by DNP might lead to a higher steady-state level of ADP and P_i in the cytoplasm and thus favour the anaerobic glycolysis. The following experiments have been conducted in order to obtain support for this hypothesis.

Effect of DNP, KCN and methylene blue, alone or incombination, on the glycolysis of the S_3A ascites carcinoma

First, it was checked, by inhibition of the cytochrome oxidase in the presence of KCN, whether the conditions were really strictly anaerobic. Aerobic glycolysis in the presence of KCN (10^{-3} M) was indeed found to be similar to the anaerobic glycolysis in the presence or absence of KCN (Table IV; Experiments 1*c*, 2*c* and 4*c*). The effect of DNP on the aerobic and anaerobic glycolysis was also apparent in the presence of KCN (Experiments 2*d*, 4*d*). According to expectation methylene blue only abolished the Pasteur effect (Experiments 1*d*, 1*e*, 3*e*).

Anaerobic glycolysis in homogenates of the S_3A ascites carcinoma

Homogenates were prepared as mentioned under Materials and Methods. Microscopic examination showed that at the most 10 per cent of the cells had remained intact. Moreover, intact cells did not metabolize added fructose diphosphate but the homogenates were very active in this respect.

In all the following experiments incubation was performed in the medium according to LePage (1948) from which fructose diphosphate was omitted. The medium designated with the capital A contains KF (10^{-2} M) and pyruvate to circumvent the enolase block, whereas in medium B fluoride was absent and only a trace amount of pyruvate was added to prime the triose phosphate dehydrogenase reaction by affording a rapid reoxidation of DPNH. In both media glycolysis proceeded from glucose. A trace amount of ATP was also present to prime the hexokinase reaction; when no ATP was added glycolysis still proceeded, even in medium B, at a high rate. The latter result indicated that enough ATP was initially present to satisfy the hexokinase reaction and, thus, that ATP dephosphorylation was not very high. The ease of obtaining a vigorous glycolysis with ascites cell homogenates under the above conditions stands in marked contrast to the situation in other homogenates (*e.g.*, from liver) in which it is necessary to add ATP, fructose diphosphate and/or hexokinase to obtain similar results.

TABLE IV.—*Effect of* 2: 4-*Dinitrophenol, Potassium Cyanide and Methylene Blue Alone or in Combination on the Glycolysis of* S_3A *Ascites Carcinoma Cells*

DNP: 10^{-4} M, KCN: 10^{-3} M, Methylene blue: 10^{-4} M.
Incubation period: Experiments 1, 2, 3 and 4: 45, 30, 45 and 30 minutes; mg. of dry weight cells: 6·1, 5·4, 6·1 and 6·4, respectively.
Lactate production in μmoles.

Experiment No.	Addition	Lactate production	
		Anaerobic	Aerobic
1*a*	None (control)	12·5	9·4
b	DNP	20·2	21·3
c	KCN	12·2	11·9
d	M Bl	—	11·9
e	KCN + M Bl	—	11·7
2*a*	None (control)	7·3	5·0
b	DNP	18·9	20·0
c	KCN	—	7·2
d	KCN + DNP	—	16·6
3*a*	None (control)	12·3	7·5
b	DNP	22·3	23·9
c	M Bl	—	12·1
4*a*	None (control)	10·4	7·1
b	DNP	17·1	16·9
c	KCN	10·8	9·9
d	KCN + DNP	17·0	17·9

In the chain of reactions which starts with one molecule of glucose and ends with two molecules of lactic acid, two molecules of ATP are needed to phosphorylate glucose to fructose-1,6-diphosphate and in subsequent reactions four molecules of ATP are generated: 2 ATP from 2 P_i and 2 ADP by the triose phosphate dehydrogenase system, and 2 ATP from 2 ADP and the phosphate contained in 2 molecules of enolphosphopyruvate. For the purpose of the present discussion the glycolytic reactions may be abbreviated to the following scheme, in which A and B denote the reactions occurring in medium A and B:

$$\left.\begin{array}{l}\text{Glucose} + \text{ATP} \xrightarrow{\text{(A, B)}} \text{Glucose-6-P} + \text{ATP} \\ \text{Fructose-6-P} + \text{ATP} \xrightarrow{\text{(A, B)}} \text{Fructose-di-P} + \text{ADP}\end{array}\right\} 2\ \text{ATP} \rightarrow 2\ \text{ADP}$$

$$2\left\{\begin{array}{l}\left.\begin{array}{l}\text{3-P-glyceraldehyde} + P_i + \text{ADP (+ DPN)} \xrightarrow{\text{(A, B)}} \\ \quad\text{3-P-glycerate} + \text{ATP (+ DPNH}_2\text{)}\end{array}\right\} 2\ P_i + 2\ \text{ADP} \rightarrow 2\ \text{ATP} \\ \text{2-P-glycerate} \xrightarrow[\text{(B)}]{\text{blocked by KF}} \text{enolphosphopyruvate} \\ \left.\begin{array}{l}\text{enolphosphopyruvate} + \text{ADP} \xrightarrow{\text{(B)}} \text{enolpyruvate} + \text{ATP} \\ \text{(enol)pyruvate (+ DPNH}_2\text{)} \xrightarrow{\text{(A, B)}} \text{lactate (+ DPN)}\end{array}\right\} 2\ \text{ADP} \rightarrow 2\ \text{ATP}\end{array}\right.$$

Glycolysis must come to a stop as soon as the available P_i and ADP is converted to ATP. It should, however, be noted that the reaction rate of the triose phosphate

dehydrogenase system decreases already before the P_i and ADP have disappeared completely. The ATPases which split ATP into ADP and P_i and other ATP-consuming processes, are thus important in conditioning the rate of glycolysis. If these activities are very low, glycolysis will proceed sluggishly and soon stop. On the other hand, if ATP dephosphorylation is very high the kinases become deprived of ATP and glycolysis will stop also. Such a situation was encountered by Meyerhof and Wilson (1949) in homogenates of certain solid tumours. Since the kinases and the ATPases compete for ATP, the relative activity of these enzymes will govern the glycolytic rate. It appeared that the hexokinase was very

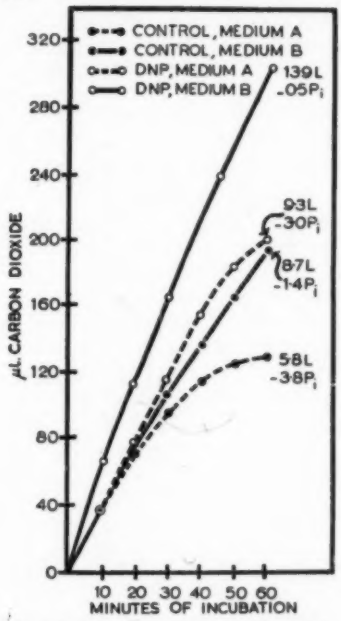

FIG. 1.—The effect of 2 : 4-dinitrophenol on the anaerobic glycolysis of a S_3A ascites carcinoma cell homogenate.

Homogenate corresponding with 6·7 mg. of dry weight cells. 10^{-4} M DNP added as indicated. Medium A and B compare text. L and P_i denote µmoles lactate produced and µmoles inorganic phosphate taken up (−), respectively. 3·9 µmoles P_i were present at the start.

active in homogenates of the S_3A ascites carcinoma and that ATP dephosphorylation was not very high. It follows then that in medium A, which contains the ATPase inhibitor fluoride, anaerobic glycolysis will proceed more slowly than in the absence of the inhibitor (medium B). This was always found to be the case. From Fig. 1, which represents one of these experiments, it can be seen that the anaerobic glycolysis in medium A began to slow down after 20–30 minutes and came to a stop after 50 minutes, whereas in medium B glycolysis still continued unhampered. Measurement of the inorganic phosphate showed that in medium A the concentration of P_i had dropped to a low value (0·2 µmole P_i left from the 3·9 initially present) whereas in medium B, in spite of the higher lactate production,

much more P_i (2·5 μmoles) was left. These results strongly suggest that the rate of glycolysis was governed by the available P_i (and ADP), and that, in the presence of fluoride, P_i soon reached a limiting concentration by being continuously converted to 2-phosphoglycerate.

TABLE V.—*The Effect of P_i, ADP and DNP on the Anaerobic Glycolysis of Homogenates of the S_3A Ascites Tumour*

All quantities in micromoles.

Experiment No.	Medium (dry weight of cells; time of incubation)	P_i/ADP	DNP (10^{-4} M)	Lactate production	Extra lactate following addition of P_i	Extra lactate following addition of ADP	Extra lactate following addition of DNP	P_i uptake
1*a*	A (8 mg; 60′)	3·9/—	—	10·4				3·8
b		3·9/—	+	15·5	6·5		5·1	2·6
c		10·9/—	—	16·9				6·2
d		10·9/—	+	16·1		−0·6	0·8	5·3
e		10·9/9·6	—	16·3				7·6
f		10·9/9·6	+	16·6			0·3	6·2
2*a*	A (6·8 mg.; 80′)	3·9/—	—	11·4				—
b		3·9/—	+	16·1	5·1		4·7	—
c		10·9/—	—	16·5				—
d		10·9/—	+	17·6		1·2	1·1	—
e		10·9/9·6	—	17·7				—
f		10·9/9·6	+	19·1			1·4	—
3*a*	B (9·0 mg.; 70′)	3·9/—	—	17·6				1·9
b		3·9/—	+	26·2	4·6		8·6	1·2
c		10·9/—	—	22·2				3·9
d		10·9/—	+	26·4		6·6	4·2	3·3
e		10·9/9·6	—	28·8				5·8
f		10·9/9·6	+	30·1			1·3	5·2
4*a*	B (8·7 mg.; 60′)	3·9/—	—	16·1				1·9
b		10·9/—	—	20·0	3·9			3·6
c		10·9/9·6	—	25·8		5·8		6·2
5*a*	B (5·2 mg.; 40′)	3·9/—	—	4·3				1·4
b		3·9/—	+	8·7			4·4	0·4
c		7·6/9·6	—	8·1				2·4
d		7·6/9·6	+	9·4			1·3	0·8
6*a*	A (9 mg.; 60′)	3·9/—	—	7·8				3·1
b		10·3/—	—	15·3	7·5	1·6		6·6
c		3·9/9·6	—	9·4		−0·6		2·9
d		10·3/9·6	—	14·7	5·3			5·7
e	B (*ditto*)	3·9/—	—	14·4				1·1
f		10·3/—	—	18·9	4·5	4·5		2·3
g		3·9/9·6	—	18·9		3·4		1·1
h		10·3/9·6	—	22·3	3·4			2·1

If this interpretation is correct it should be possible, first, to enhance the anaerobic glycolysis in the presence of fluoride by adding extra P_i to medium A. This was indeed found to be possible (Table V; Experiments 1*a–c*, 2*a–c*, 6*a–b*; compare also Emmelot and Bos, 1958*a*). Secondly, addition of ADP might have little extra effect on lactate production, since the necessary ADP for the triose phosphate dehydrogenase system is provided by the active kinases, whereas ADP is no longer required for the phosphorylating reaction in which enolphospho-

pyruvate participates because fluoride blocks the enolase reaction and no enolphosphopyruvate can be formed. ADP was indeed found to have little effect on the rate of the anaerobic glycolysis in medium A regardless of the P_i concentration (Table V ; Experiments 1*c–e*, 2*c–e*, 6*b–d*, 6*a–c*).

It appeared that the rate of the anaerobic glycolysis could also be enhanced in medium B by addition of P_i (Table V ; Experiments 3*a–c*, 4*a–b*, 6*e–f*), though, as might be expected, to a smaller extent than in medium A. The glycolysis might be more dependent upon ADP in the absence than in the presence of fluoride, since in the former case the conversion of enolphosphopyruvate to enolpyruvate requires ADP. The stimulatory effect of ADP in medium B was consistently observed, not only at high but also at low P_i concentrations (Table V ; Experiments 3*c–e*, 4*b–c*, 6*f–h* and 6*e–g*, respectively.)

Effect of 2: 4-*dinitrophenol on the anaerobic glycolysis of* S_3A *ascites cell homogenates*

Fig. 1 and Table V show that the anaerobic glycolysis could also be stimulated by adding DNP (10^{-4} M), instead of P_i and ADP, to the homogenates incubated in medium A or B (Table V, in the presence of KF : Experiments 1*a–b*, 2*a–b* ; in the absence of KF : Experiments 3*a–b*, 5*a–b*). The enhanced lactate production in the presence of DNP was always accompanied by a diminished uptake of P_i. It may be concluded that, as a result of the DNP-activation of the ATPases, the higher P_i or P_i plus ADP concentration (medium A and B, respectively) allowed a more active glycolysis. If so, DNP should be without effect when extra P_i (and ADP) is added to the media. This was actually found to be so as shown in column 8 of Table V (Experiments 1, 2, 3 and 5) ; that the anaerobic glycolysis in medium A was only dependent upon the P_i concentration but in medium B also upon the ADP concentration, is shown by the fact that in the former medium DNP had no effect when the P_i concentration was raised (Experiments 1*c–d*, 2*c–d*) whereas under similar conditions DNP still activated the glycolysis in medium B (Experiments 3*c–d* and similar results), but only to a small extent when extra ADP was added (Experiments 3*e–f*, 5*c–d*).

TABLE VI.—*Effect of DNP on ATP-dephosphorylation by Intact* S_3A *Ascites Carcinoma Cells.*

Conditions: Emmelot *et al.* (1959)

Dry weight of cells (mg.)	Temperature of incubation	DNP (10^{-4} M)	µg. P released after (minutes) 5	10	20
3·6	27°	−	4	10	27
		+	9	15	28
5·8	27°	−	15	20	30
		+	21	30	40
3·8	37°	−	14	25	45
		+	21	35	58

Effect of 2: 4-*dinitrophenol on ATP dephosphorylation by homogenates and mitochondria of the* S_3A *ascites carcinoma*

It has been shown previously (Emmelot *et al.*, 1959) by measurement of the P_i release from added ATP that DNP actually increases the ATP-dephosphorylation by the ascites cell homogenates and mitochondria isolated therefrom, both at

27° and 37° C. In the case of the homogenates, the enhancement of the phosphate release was not due to an effect mediated by the small amount of intact cells which were still present. Intact cells incubated in the presence or absence of DNP at 27° and 37° C. did not release any phosphate, but did so in the presence of ATP. The latter dephosphorylation was but slightly enhanced by DNP (Table VI). It has been reported (Acs, Ostrowski and Straub, 1954) that ascites cells contain an ATPase located at the outer membrane. If one assumes that ATP is unable to penetrate into the intact cells, the present results suggest that the extracellular-bound ATPase is but little activated by DNP.

TABLE VII.—*Effect of Stilboestrol and Thyroxine on the ATP-dephosphorylation by Mouse Liver Mitochondria and S_3A Ascites Tumour Homogenate at 37° C.*

Stilboestrol 5×10^{-5} M; thyroxine $7{\cdot}5 \times 10^{-5}$ M. Mitochondria corresponding with 25 mg. wet weight of mouse liver, ascites homogenate corresponding with 3 mg. dry weight of cells.

Enzyme source	Addition	μg. P released after (minutes)		
		5	10	20
Liver mitochondria	—	22	30	35
	Stilboestrol	41	62	105
	Thyroxine	36	50	93
Ascites tumour homogenate	—	25	42	60
	Stilboestrol	35	82	132
	Thyroxine	32	73	150

TABLE VIII.—*Effect of Stilboestrol on the Glycolysis of Intact and Homogenized Ascites Tumour Cells*

Incubation during 45–60 minutes at 37° C.

Preparation	Addition	Lactate production (micromoles)	
		Anaerobic	Aerobic
Intact cells (8·2 mg.)	—	16·8	10·9
	Stilboestrol	17·8	16·7
Intact cells (6·1 mg.)	—	10·5	..
	Stilboestrol	16·5	..
Homogenate (9·0 mg.; medium B)	—	·13·9	..
	Stilboestrol	19·4	..

Effect of Stilboestrol and Thyroxine on Glycolysis and ATP dephosphorylation of intact S_3A ascites cells and homogenates

Stilboestrol and thyroxine had no effect on the latent ATPase activity of mouse liver mitochondria when incubation was carried out at 27° C. However, at 37° C. a marked stimulation of the ATPases of mitochondria and ascites cell homogenates was observed (Table VII). (The activation of rat liver mitochondrial ATPase by thyroxine at 37° C. has also been observed by Maley and Johnson (1957).) The Pasteur effect was completely abolished by stilboestrol (5×10^{-5} M) in all experiments (Table VIII). The anaerobic glycolysis of the ascites cells was enhanced (1·5–1·8-fold) by stilboestrol only in a number of experiments with the smaller amounts of cells. In homogenates a rise of 1·4–1·5-fold in the anaerobic

glycolysis was noted in the presence of stilboestrol. The oestrogen inhibited the oxidation of the ascites cells by approximately 30 per cent in the present experiments (Shacter, 1956 ; Emmelot and Bos, 1958*b*).

The observations of Barker and Lewis (1956) that thyroxine may enhance the aerobic and anaerobic glycolysis of the Ehrlich ascites carcinoma has been confirmed in the present experiments with the S_3A ascites carcinoma. The results were, however, irregular, probably as a result of the decreased solubility of the hormone after slight pH changes of the medium.

Effect of 2: 4*-dinitrophenol on the incorporation of* [1–^{14}*C*]*leucine into* S_3A *ascites carcinoma protein under aerobic and anaerobic conditions*

If DNP activates the ATPases of the mitochondria of intact ascites tumour cells, it may be expected that the steady-state concentration of glycolytic ATP is lower in the presence of DNP than in its absence. This lowered ATP concentration did not inhibit the hexo- and fructokinase reactions, but might have an effect upon ATP-dependent synthetic processes such as amino acid incorporation into proteins. The following experiments (Table IX) show this to be the case. The incorporation of [1–^{14}C]leucine into the proteins of the ascites cells was of the same order in the presence or absence of glucose under *aerobic* conditions. Mitochondrial ATP, generated by the endogenous respiration, could thus completely satisfy the energy requirements of the amino acid incorporation processes (compare Emmelot and van Vals, 1957). Addition of DNP inhibited leucine incorporation, while addition of glucose counteracted the latter inhibition but could not restore the rate of incorporation reached in the absence of DNP. Incorporation of leucine under *anaerobic* conditions was dependent upon the presence of glucose (glycolytic ATP). With glucose present, addition of 10^{-4} M DNP consistently resulted in an inhibition of about 50 per cent of the anaerobic amino acid incorporation. Similar results were obtained with [1–^{14}C]leucine added in the range of 0·3–1·0 μmole.

It is of interest to compare the present results with those obtained with the Ehrlich ascites carcinoma. Since the anaerobic glycolysis of the Ehrlich ascites carcinoma was stimulated by DNP in our experiments (Table III), the amino acid incorporation into the protein of these cells should be depressed under the latter conditions. It has actually been reported that DNP, under anaerobic conditions and in the presence of glucose, inhibited the amino acid incorporation into the latter cells by 30 per cent (Rabinowitz, Olson and Greenberg, 1955). However, Bickis, Creaser, Quastel and Scholefield (1957) observed only a slight inhibitory effect. The latter result might have been due to the " quality " of their cells ; cells which are not in an optimal condition (e.g. as a result of repeated washings) might already show activated ATPases.

Our hypothesis demands that in those cases in which DNP does not enhance the anaerobic glycolysis, the amino acid incorporation under anaerobic conditions in the presence of glucose should not be affected either. This was found to be so in experiments carried out with slices of the mouse sarcoma UV 256 (Table IX ; Experiment 6).

COMMENT

The present results furnish strong evidence for the supposition that the anaerobic glycolysis of ascites tumour cells may be raised by activation of the intracel-

lular, probably mitochondrial, ATPases. The ascites cells apparently possess an enormous capacity to glycolyse, which under normal conditions cannot be attained anaerobically as a result of the relatively slow ATP-dephosphorylation. From the experiments on leucine incorporation and those conducted with the homogenates it is concluded that under anaerobic conditions the ATP-turnover increased and its steady-state concentration decreased when DNP was present.

TABLE IX.—*Effect of* 2: 4-*Dinitrophenol on the Incorporation of* [1–^{14}C] *Leucine into the Proteins of the* S_3A *Ascites Carcinoma under Aerobic and Anaerobic Conditions in the Presence and Absence of Glucose*

Incubation in Krebs-Ringer phosphate(*) or bicarbonate(**) buffer aerobically and anaerobically (gas phase: 100% O_2 and 100% N_2; 95% O_2 + 5% CO_2 and 95% N_2 + 5% CO_2, respectively), DL-[1–^{14}C]-leucine (0·28 μmoles containing 0·33 μc).

Experiments 1, 2: 300 mg. wet weight of cells in 5 ml. 0·02 M phosphate buffer, 6 mg. glucose, 60 minutes.

Experiments 3, 4: 200 mg. wet weight of cells in 5 ml. 0·02 M phosphate (or HCO_3) buffer, 3 mg. glucose, 30 minutes.

Experiment 5: 200 mg. wet weight of cells in 6 ml. 0·03 M phosphate (or HCO_3) buffer, 6 mg. glucose, 30 minutes.

Experiment 6: 300 mg. wet weight of slices of sarcoma UV 256 in 3 ml. buffer, 3 mg. glucose, 30 minutes.

Experiment No.	Glucose	DNP	Protein specific activity (c./m.) Anaerobic incubation (*)	Anaerobic incubation (**)	Aerobic incubation (*)	Aerobic incubation (**)
1, 2	−	..	84	..	870	..
	−	$5 \cdot 10^{-5}$	31	..	445	..
	−	10^{-4}	14	..	206	..
	+	..	907	..	945	..
	+	$5 \cdot 10^{-5}$	497	..	537	..
	+	10^{-4}	339	..	421	..
3, 4	−	..	40	74	..	..
	−	10^{-4}	10	15	..	..
	+	..	588	812	..	..
	+	10^{-4}	266	427	..	..
5	−	..	45	81	629	715
	−	10^{-4}	20	..	106	124
	+	..	639	859	576	708
	+	10^{-4}	326	428	315	371
6§	−	..	..	35	..	..
	−	10^{-4}	..	35	..	..
	+	..	..	314	..	..
	+	10^{-4}	..	322	..	..

§ Experiment carried out with slices of the mouse sarcoma UV 256.

In bone marrow cells, non-malignant and malignant lymphoblasts and slices of solid tumours no effect of DNP on the anaerobic glycolysis could be demonstrated. A variety of reasons may be responsible for this lack of effect. The glycolytic capacity may be smaller and the relative rate of ATP-dephosphorylation may be higher in the latter cells and tissues than in the ascites cells. Furthermore

the rate of penetration of glucose may limit the glycolysis. In the case of tissue slices mechanical factors may be involved. Since the entrance of glucose may occur by an active transport mechanism, the number and the specific activity of the active sites in the cell membrane may also be of importance.

It has been shown that glucose penetrates into ascites tumour cells very rapidly and that the rate of glycolysis is not governed by the rate of glucose entrance (Crane, Field and Cori, 1957 ; Cori, 1956). The generally high glycolysis of ascites as well as of solid tumour cells may be due to this phenomenon, the relative differences between the two types being dependent on the unicellular or multicellular organisation (Hechter, 1957; Bloch-Frankenthal and Weinhouse, 1957).

It has been suggested (Lynen, 1958) that glucose uptake is dependent upon glycolytic ATP. The decreased aerobic glucose phosphorylation has been explained (Chance and Hess, 1956 ; Lynen, 1958) by assuming that the ATP which is generated under aerobic conditions in the mitochondria is retained and does not readily equilibrate with the cytoplasm. As a result the hexo- and fructokinase of the glycolytic pathway should be inhibited as compared with the situation under anaerobic conditions or under aerobic conditions with DNP present, in which more ATP is formed in the cytoplasm. However, the finding that the anaerobic glycolysis of the ascites cells can be markedly stimulated by increasing the dephosphorylation of glycolytic ATP, indicates that neither the uptake of glucose nor its phosphorylation is impaired by decreasing the steady-state concentration of glycolytic ATP of ascites tumour cells under anaerobic conditions.

SUMMARY

1. The effect of DNP on the aerobic and anaerobic glycolysis of ascites tumour cells, tumour slices and several types of normal and malignant cell suspensions has been studied.

DNP abolished the Pasteur effect in all cases. The anaerobic glycolysis of the Ehrlich and S_3A ascites carcinomas and the T 86157 ascites lymphosarcoma was found to be stimulated by DNP (10^{-4} M) about 2-fold in general, but higher increases were also observed occasionally. No effect of DNP on the anaerobic glycolysis of tumour slices (including the solid T 86157 lymphosarcoma), (non-) malignant lymphoblasts (derived from T 86157 by subculture *in vitro*) and bone marrow cells was observed. The anaerobic glycolysis of cell suspensions prepared from the solid T 86157 lymphosarcoma was enhanced by DNP under certain conditions.

2. The anaerobic glycolysis of homogenates prepared from the S_3A ascites carcinoma was markedly dependent upon the concentration of P_i or P_i plus ADP, depending on the experimental conditions. A significant rise in the anaerobic lactate production and a drop in the P_i uptake by the homogenates in the presence of DNP was observed. DNP exerted no effect when the concentration of P_i (and ADP) in the medium was raised.

3. DNP stimulated ATP-dephosphorylation by homogenates and isolated mitochondria of the ascites tumour cells, but not of intact ascites tumour cells, both at 27° and 37° C.

4. Stilboestrol had a similar, though smaller, effect as DNP.

5. The energy (ATP) derived from the endogenous (fatty acid) oxidation of the ascites tumour cells was equivalent to that derived from aerobic glycolysis plus glucose oxidation, or from the anaerobic glycolysis, in sustaining amino

acid incorporation into the proteins of the ascites cells. DNP inhibited amino acid incorporation into the protein of the S_3A ascites carcinoma under anaerobic conditions in the presence of glucose.

6. It is concluded that under anaerobic conditions, when nearly all the P_i and ADP is available to the cytoplasmic glycolysis, the rate of glycolytic-ATP generation by the ascites tumour cells is higher than the rate of ATP-dephosphorylation ; as a result the anaerobic glycolysis operates below its potential capacity. In the presence of DNP the mitochondrial ATPases are activated and the higher steady-state concentrations of P_i and ADP, resulting from the splitting of glycolytic ATP by the activated mitochondrial ATPases, allow glycolysis to proceed at a higher rate than in the absence of DNP. However, since the increased ATP turnover in the presence of DNP induces a fall in the steady-state concentration of ATP, ATP-dependent anabolic processes of the ascites tumour cells, such as amino acid incorporation, are inhibited under anaerobic conditions in the presence of glucose and DNP.

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REFERENCES

Acs, G., Ostrowski, W. and Straub, F. B.—(1954) *Acta physiol. hung.*, **6**, 261.
Barker, S. B.—(1957) ' Methods in Enzymology ' Ed. by S. P. Colowick and N. O. Kaplan. New York (Academic Press) vol. **3**, 241.
Barker, S. B. and Lewis, W. J.—(1956) *Proc. Soc. exp. Biol. N.Y.*, **91**, 650.
Bickis, I. J., Creaser, E. H., Quastel, J. H. and Scholefield, P. G.—(1957) *Nature, Lond.*, **180**, 1109.
Bloch-Frankenthal, L. and Weinhouse, S.—(1957) *Cancer Res.*, **17**, 1082.
Chance, B. and Hess, B.—(1956) *Ann. N.Y. Acad. Sci.*, **63**, 1008.
Clowes, G. H. A. and Keltch, A. K.—(1954) *Proc. Soc. exp. Biol. N.Y.*, **86**, 629.
Cori, C. F.—(1956) ' Currents in Biochemical Research.' Ed. by D. E. Green. New York (Interscience), p. 198.
Crane, R. K., Field, R. A. and Cori, C. F.—(1957) *J. biol. Chem.*, **224**, 649.
Emmelot, P. and Bos, C. J.—(1955) *Rec. Trav. chim. Pays-Bas*, **74**, 1343.—(1958*a*) *Biochem. Pharmacol.*, **1**, 105.—(1958*b*) *Exp. Cell. Res.*, **14**, 132.—(1959) *Brit. J. Cancer*, **13**, 520.
Idem, Bos, C. J., Brombacher, P. J. and Hampe, J. F.—(1959) *Ibid.*, **13**, 348.
Idem and van Vals, G. H.—(1957) *Ibid.*, **11**, 620.
Fiske, C. H. and Subbarow, Y.—(1929) *J. biol. Chem.*, **81**, 629.
Freund, E. and Kaminer, G.—(1910) *Biochem. Z.*, **26**, 312.
Hechter, O.—(1957) *Cancer Res.*, **17**, 512.
LePage, G. A.—(1948) *J. biol. Chem.*, **176**, 1009.
Lynen, F.—(1956) *Int. Congr. Biochem.*, Brussels, 1955, **3**, 294.—(1958) ' Neuere Ergebnisse aus Chemie u. Stoffwechsel der Kohlenhydrate,' Berlin (Springer Verlag), p. 155.
Maley, G. F. and Johnson, D.—(1957) *Biochem. biophys. Acta*, **26**, 522.
Meyerhof, O. and Wilson, J. R.—(1949) *Arch. Biochem.*, **21**, 1, 22.
Rabinowitz, M., Olson, M. E. and Greenberg, D. M.—(1955) *J. biol. Chem.*, **213**, 1.
Racker, E.—(1956) *Ann. N.Y. Acad. Sci.*, **63**, 1017.
Shacter, B.—(1956) *J. nat. Cancer Inst.*, **16**, 1453.
Simon, E. W.—(1953) *Biol. Rev.*, **28**, 453.
Stickland, L. H.—(1956) *Biochem. J.*, **64**, 503, 515.

THE CONTENT OF DIPEPTIDASES AND ACID PROTEINASES IN THE ASCITIC FLUID OF MICE WITH ASCITES TUMOURS

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ATTEMPTS are being made to investigate whether proteolytic enzymes, possibly liberated from the tumour cells, are implicated in the observed extracellular proteolysis of fibrous host proteins at the periphery of malignant tumours (Sylvén, 1945 and 1954; Sylvén and Malmgren, 1957). The solid mouse tumour materials did not, until very recently, allow separate sampling of the extracellular fluid for enzymic assays. Therefore, the ascites tumour material was chosen since in this case an extracellular fluid compartment could easily be sampled. The present line of approach is supported by some recent evidence that malignant cells in general are more permeable than normal non-malignant cells in the sense that cytoplasmic proteins and enzymes are more freely passing out of the cell membranes. The particular physiological conditions and states of adaptation favouring such a liberation are not yet understood.

This report summarizes serial data on the total "over-all" dipeptidase and acid proteinase activities of (*a*) the ascites tumour cells, (*b*) the cell-free ascites fluid, and (*c*) the corresponding blood plasma activity levels in the same tumour-bearing mice. The particular acid proteinases of normal blood plasma active in the pH range 2 to 6 have been tentatively characterized as belonging to the pepsin and cathepsin groups (Mirsky *et al.*, 1952). The changes in plasma dipeptidase and acid proteinase activities in the course of ascites tumours have been described (Ottoson and Sylvén, 1959). The present results are, however, burdened by several limitations restricting our conclusions. In view of the transport problems involved, the expected more or less non-specific host reactions, and the dilution effects in the different compartments, more direct evidence is needed as to the basic permeability problems of the tumour cells referred to above.

PREVIOUS DATA ON THE PERTINENT ENZYMIC ACTIVITIES

Blood serum and plasma.—It is well known that normal plasma contains a considerable pool of various peptidases active against commonly tested di- and tripeptides (Grassmann and Heyde, 1929; Fruton, 1946; Fleisher and Butt, 1953).

The dipeptidase activity of mouse plasma against alanylglycine (AG) undergoes a continuous increase in the course of ascites tumour growth (Ottoson and Sylvén, 1959). An endopeptidase activity in rabbit serum against benzoylglycineamide at pH 5·4 was further described by Fruton (1946). In addition, normal mouse plasma contains at least two types of proteinases with activity optima against

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urea-denatured haemaglobin at pH 3·5 and 4·5 (Fig. 3). These proteinase activities undergo characteristic changes in the course of ascites tumour growth (Ottoson and Sylvén, 1959).

The total dipeptidase activity referred to above is of a moderate magnitude in mouse plasma (about 0·2 μl. 0·1 M. NaOH per μl. plasma per hour), while the proteinase activities at pH 3·5 and 4·5 are high (extinctions about 0·15 per μl. per 90 minutes at 37°) as compared with those of human plasma. The cellular sources of these enzymes are not known in detail. It seems likely that the proteinase activity at pH 3·5 is due to pepsinogen (activated) from the stomach mucosa (Mirsky *et al.*, 1952).

Lymph.—Lymph enzymes in general present lower concentrations per volume than blood plasma (Yoffey and Courtice, 1956). The concentration of enzymes is said to run largely parallel with the concentration of proteins in the larger lymph trunks so far investigated. A trypsin-like proteinase in thoracic duct lymph of the dog was described by Osato (1921), and a dipeptidase in rabbit leg lymph by Ishino (1935/38). The enzymic content of the smaller efferent lymph channels is not known; however, according to Doyle (1955), lymph from the appendix has a slightly higher peptidase level than blood plasma. No data on the content of acid proteinases are available.

Interstitial tissue fluid.—Little is known of the enzymal activities in the interstitial fluid of normal tissues. The concentrations are expected to be of a similar order of magnitude as that in lymph. An aminopeptidase activity has been observed by histochemical methods in the oedema fluid of skin disorders in man (Braun-Falco, 1957), and also at the periphery of several malignant tumours (Burstone, 1956). A proteinase activity was long ago postulated to occur in the extracellular fluid of healing wounds explaining the removal of preformed fibrous proteins. It is not known, however, whether these activities are due to cell damages or if they occur also under normal *in vivo* conditions. It was cursorily mentioned by Libenson and Jena (1957) that the cell-free tissue fluid within certain transplanted human tumours contained a greater proteolytic activity at pH 4 than the normal subcutaneous tissue fluid distant from the tumour site. Quantitative assays of the peptidase and catheptic activities of the extracellular fluid taken *in vivo* from various solid mouse tumour transplants (Sylvén, unpublished) distinctly indicate that these activities per unit volume are considerably higher than the levels observed in normal lymph and plasma.

Preliminary data on the normal peritoneal fluid collected by capillary techniques from the intact peritoneal cavity of the same strain of inbred mice, as used in this report, are being collected. Samples pooled from a large number of mice showed a rather high total dipeptidase figure (about 4 times the corresponding plasma activity). The pH 3·5 and 4·5 proteinase activities against urea-denatured haemoglobin were of the same order as those of the corresponding blood plasma. The relative activities are for comparison included in Fig. 7.

Ascites tumour fluid.—The dipeptidase activity in the fluid of mouse ascites tumours was reported to be low (Malmgren, Sylvén and Révész, 1955). The presence of a carboxypeptidase inhibitor in the fluid was demonstrated by Feinstein and Ballin (1953), and later on also noted against the AG peptidase by Malmgren, Sylvén and Révész (1955). With reference to the proteinase activities in ascites tumour fluid little is known. Spontaneous hydrolysis (pH unknown) of the inherent proteins did not occur during two hours' incubation time according

to Christensen and Riggs (1952); this possibly excludes the presence of marked tryptic activities. Malmgren, Sylvén and Révész (1955) cursorily mentioned that a low proteinase activity was noted against nitrocasein and edestin at pH 5·3 and 3·8, respectively. The method used at that time was rather insensitive and therefore no significant changes in the proteinase activity were noted in the course of the ascites tumour growth. More data are available on the presence of other hydrolytic and glycolytic enzymes in the ascites fluid (Warburg and Christian, 1943; Kun, Talalay and Williams-Ashman, 1951; Schade, 1953; Wu and Racker, 1958; Bosch, 1958).

Ascites tumour cells.—The peptidase activities against four tested dipeptides and one tripeptide have been described in homogenates from three different ascites tumour cell strains (Malmgren, Sylvén and Révész, 1955). Serial data on the tumour cells at different times after inoculation indicated that the dipeptidase activity was proportional to the protein content of the cells. During the same time the average catheptic activity per tumour cell at pH 3·8 against acid-denatured haemoglobin remained low and fairly constant. A more detailed study of the cellular proteinases of tumours was recently published (Sylvén and Malmgren, 1957). The present report supplements previous ascites tumour data mentioned above.

MATERIALS AND METHODS

Mice.—Male backcross mice were used, produced by mating males of the DBA strain with (C3H × DBA)F_1 hybrid females. The animals were 3 to 4 months old and weighed 16–22 g. They were given a standard compressed diet and drinking water *ad libitum*.

Tumour strain.—The hyperdiploid Ehrlich ascites tumour was employed, referred to as ELD (Bayreuther, 1952; Hauschka *et al.*, 1957). The tumour has been maintained by weekly serial ascitic transfers in DBA × (DBA × C3H) backcross mice. The transfers were carried out routinely by injecting 0·1 ml. of the undiluted ascitic fluid intraperitoneally.

Measurement of ascites tumour growth.—The total volume of peritoneal fluid and the total number of tumour cells was measured by determining the dilution of an intraperitoneally injected dye (bromosulphthalein), together with total and differential cell counting and determination of the packed cell volume. The details of this procedure have been described by Révész and Klein (1954).

Sampling.—Each experiment was performed with ascites fluid and cells pooled from three identically treated mice in such a way that each mouse contributed an equal volume of ascites fluid and an equal number of cells. Pooled material was used in order to avoid most of the individual variations. The ascites fluid and cells was taken out in 3·5 per cent sodium citrate to 1/5 of the total sample volume. The fluid and the cells were separated before pooling by centrifugation for 10 minutes at about 600–800 × **g**. The first supernatant was further centrifuged for 10 minutes at about 6000 × **g** in order to remove all cell débris. Some fluid samples were carefully controlled under the phase-contrast microscope and no cell elements remained. After pooling, the supernatants were diluted 2 to 5 times with a 0·05 per cent aqueous solution of sodium deoxycholate according to our standard procedure (Sylvén and Malmgren, 1957).

The cells were washed twice in saline and then resuspended in twice their volume of saline. After counting the number of cells in each sample they were

pooled as described above. The pooled cell suspension was homogenized for 15 minutes under cooling. Sodium deoxycholate was added to obtain a suitable concentration and the mixture was left to extract for one hour at room temperature. A cell concentration corresponding to 80 $\times$ 10^6 per ml. was used for the proteinase assays and 2 to 4 $\times$ 10^6 per ml. for the dipeptidase tests.

The average ascites cell volume of the packed cells has been 25 per cent of the total ascites volume. This figure has been used for the calculation of the fluid content obtained by centrifugation.

Enzymic assays.—The dipeptidase assays by titration according to the modification of the Linderström-Lang and Holter method have been described by Sylvén and Malmgren (1957). Since the ascites peptidases may be activated by magnesium ions, $MgSO_4$ has been added to a final concentration of 0·006 M.

The proteinase determinations using urea-denatured bovine haemaglobin as a substrate have been made according to Ottoson and Sylvén (1959). All samples have been assayed both with and without the addition of cysteine to a final concentration of 0·003 M.

Since the enzymic activity data of the fluid compartments are discussed as matters of transport equilibria all results are primarily given per μl. fluid.

Protein determination.—The protein contents have been assayed in triplicate tests with a micro-Kjeldahl method. The standard deviation among tests performed on the same material was of the order of 3 per cent and the error of the mean of the three tests was of the order of 2 per cent.

RESULTS

The multiplication of the ELD ascites tumour cells was determined in two separate experiments each comprising 30 mice inoculated with 20 $\times$ 10^6 tumour cells. At daily intervals, two mice were killed and the geometric mean was calculated for each lot of total cell numbers, cell concentrations per ml. ascitic fluid, and amount of cell-free ascitic fluid (four individual determinations from two separate experiments). Some data of this series have been published earlier (Hauschka *et al.*, 1957).

Fig. 1A shows the increase in total number and concentration of free tumour cells in the ascitic fluid during 12 days of growth after inoculation. The growth of the ELD cells is characterized by a continuously increasing generation time. The decrease in the growth rate proceeds smoothly and the number of free tumour cells continues to increase until a total number of about 10^9 cells is reached. The concentration of the tumour cells per ml. fluid remains largely constant during the major part of the growth cycle and shows only a moderate random variation around a value of about 200 $\times$ 10^6 cells per ml. ascites. Fig. 1B shows the increase in the amount of the cell-free ascitic fluid. Since the mean volume of the ELD cells has been found to be fairly constant (about 1360 cubic μ) due to the constancy of the cell concentration, the increase in the amount of the cell-free ascitic fluid is directly proportional to the increase of the total number of tumour cells.

The previously reported increase in the average amount of protein per cell, as observed in the ELD and Krebs 2 ascites tumours (Malmgren, Sylvén and Révész, 1955), was corroborated also in this series of the ELD strain. The protein figure rose from an average of 1·4 μg. per 10,000 tumour cells, at the fourth day after inoculation, until about 2·8 micrograms at the eighth day, which is at variance with data by Ledoux and Revell (1955).

Protein content of blood plasma and ascites fluid.—In our stock of mice the plasma protein concentration went down from a normal level close to 6 per cent to below 4 per cent in the course of the ascites tumour growth (Fig. 2). This figure also shows that the protein concentration of the cell-free ascites fluid declines from about 4·5 per cent to about 2·5 per cent at the end of the growth

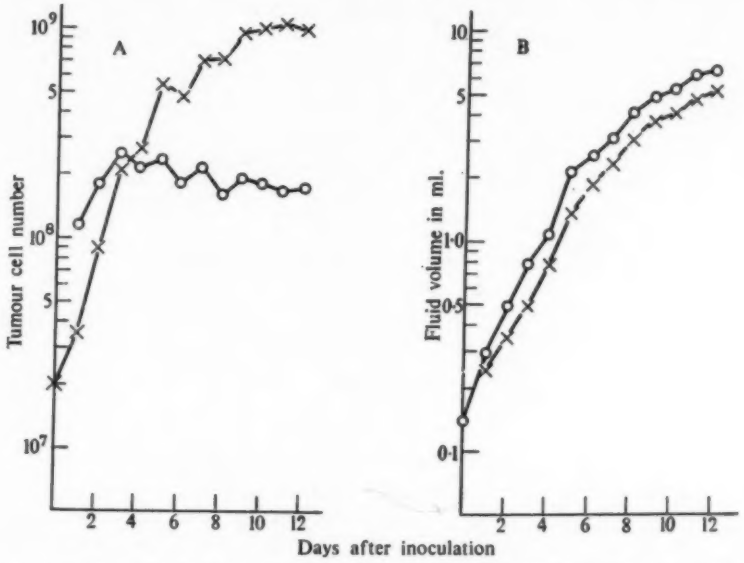

FIG. 1.—Changes in the tumour cell numbers and ascites fluid volume plotted against the time after tumour inoculation (cf. data by Hauschka *et al*,. 1957).
1, A.—Average total tumour cell numbers ×——×——×
Tumour cell concentration O——O——O
1, B.—Volume of cell-free ascites fluid ×——×——×
Total volume of ascites O——O——O

curve. Ledoux and Revell (1955) previously found in the same strain of ascites tumours an increasing protein concentration in the fluid up to about 2 per cent at the 14th day of growth. The protein content of normal rabbit lymph (hind leg) is about 2·6 per cent (Benson, Kim and Bollmann, 1955) and of human ascites fluid in cases of liver cirrhosis about 3 per cent (Schoenberger *et al.*, 1956).

Albumin-globulin ratio of the ascites fluid.—The relative amounts of albumin and globulins assayed by the electrophoretic method* were as follows :

	Albumin (%)	Globulins (%)	A/G ratio
Normal male mouse plasma (inbred stock)	43	57	0·75
Plasma of ascites tumour-bearing male mice	50	50	1·0
Ascites tumour fluid	56	44	1·25

* The determinations have kindly been made in the laboratory of Dr. B. Olhagen, at the Rheumatic Clinic, Karolinska Hospital, Stockholm.

The relative excess of the albumin fraction in the ascites fluid agrees with the figure of Kun *et al.* (1951), who found about 58 per cent albumin in pooled Ehrlich ascites tumour fluid. The A/G ratio in the ascites fluid is thus 1·25, which is significantly larger than that of normal mouse plasma.

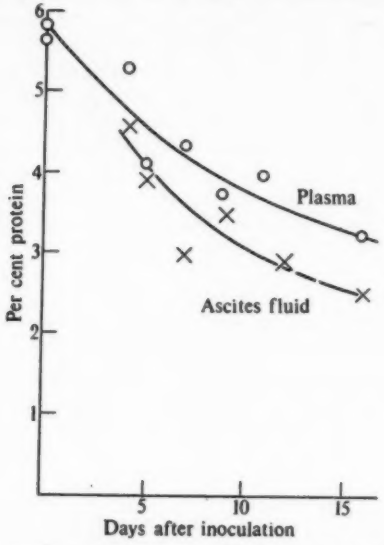

FIG. 2.—The change in protein content of mouse blood plasma and cell-free ascites tumour fluid with the time after inoculation. Each figure obtained from samples pooled from three mice (see text).

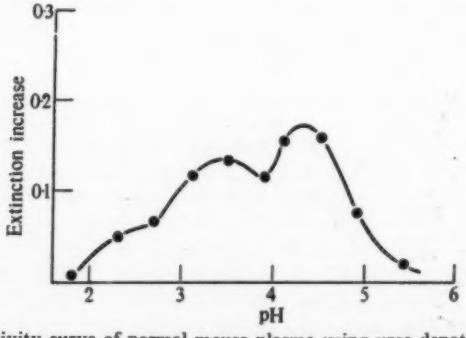

FIG. 3.—The pH-activity curve of normal mouse plasma using urea-denatured haemoglobin as a substrate ; cysteine added (see text). All extinctions are given per μl. plasma. The above activity optima will become displaced to the left when acid-denatured haemoglobin is used (Ottoson and Sylvén, 1959).

Enzymic activities of the ascites fluid and tumour cells.—From about the 12th day of ascites tumour growth the dipeptidase activity of the ascites fluid rapidly

increased (Tables I and III). This activity of ascites fluid did coincide with the late increase in dipeptidase activity of the blood plasma of the same tumour-bearing mice (Ottoson and Sylvén, 1959). Maximum activity figures (in the presence of the dipeptidase inhibitor, previously mentioned) at the end of the growth period were equivalent to about 1·8 μl. NaOH, which is more than three times the corresponding plasma level. The recorded extracellular activity constitutes from 0·5 to 4 per cent of the total activity contained per μl. ascites inclusive of the tumour cells.

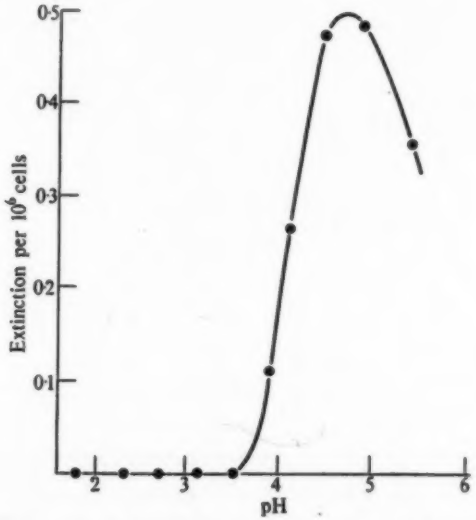

FIG. 4.—The pH-activity curve of the acid proteinases of washed ascites tumour cells pooled from three mice on the fifth day of tumour growth. Substrate urea-denatured haemoglobin; cysteine added. Extinctions expressed per 10^6 cells.

In the case of the proteinases active in the pH range of 2 to 6 the conditions are more complicated. In normal mouse plasma there are at least two (possibly three) proteinases, which show optimum activity against urea-denatured haemoglobin at pH 3·5 (3·5 enzyme) and 4·5 (4·5 enzyme) (Ottoson and Sylvén 1959; Fig. 3). The first one is probably a pepsin-like enzyme (Mirsky *et al.*, 1952), while part of the second one is expected to be of cathepsin nature. The acid proteinases of lymph have not been studied. It may further be added that the extracellular fluid obtained in diluted form from washings of the peritoneal cavity of normal mice also contains two proteinase activity peaks of about equal magnitude at pH 3·5 and 4·5 against urea-denatured haemoglobin (unpublished data). The shape of this pH-activity curve is rather similar to that of blood plasma, but distinctly different from that of the tumour ascites fluid (Fig. 5) mentioned below. In the interstitial fluid of solid mouse tumour transplants two proteinase peaks have also been observed at about pH 3·8 and another at 4·5–4·7 (Sylvén, unpublished data). The pH-dependence of the ascites tumour cell proteinases is shown in Table I and Fig. 4, which demonstrate that the pH 3·5 enzyme is lacking,

while, instead, enzymes of the cathepsin group with optimum activity around pH 4·5 are present. On the whole, the cathepsin activity is low in the tumour cells (Malmgren, Sylvén and Révész, 1955; Sylvén and Malmgren, 1957). The appearance of some pH 3·5 proteinase in the tumour cell homogenates at the end of the growth period (Table I) is considered to be caused by an admixture of proteins from the ascites fluid not removed by our saline washing.

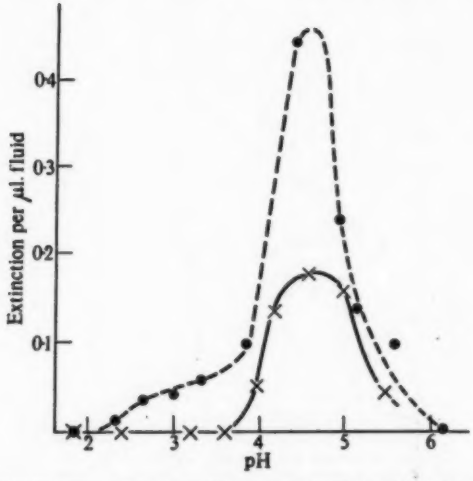

FIG. 5.—The pH-activity curve of acid proteinases contained in fresh cell-free ascites fluid. Conditions the same as in Fig. 3. Data from three pooled samples of five days old (×——×——×) and twelve days old (●- - - -●) ascites tumours.

TABLE I.—*Serial Data on the Total Dipeptidase and Proteinase Activity of Ascites Tumour Cells*

Each figure obtained on materials pooled from 3 inbred male mice.

Age of tumour (in days)	Dipeptidase activity (µl. 0·1 M NaOH per 10^4 cells	Proteinase activity Extinction increase per 10^6 cells			
		Cysteine added		Cysteine not added	
		pH 3·5 E	pH 4·5 E	pH 3·5 E	pH 4·5 E
4	—	0·15	0·30	0·02	0·32
5	—	0·00	0·94	—	—
7	0·7	0·00	0·84	0·07	0·70
9	2·9	0·00	2·10	0·14	2·16
12	2·5	0·09	0·72	0·15	0·64
16	2·0	0·14	1·46	0·23	1·60

The ascites tumour fluid, on the other hand, shows certain changes in the pH distribution curve as compared with that of normal mouse blood plasma. During the early phase of tumour growth the pH 1·8–3·5 enzymes are depressed in activity or almost lacking (Fig. 5 and 6), while a 4·5 enzyme of cathepsin-type (thermo-

and acid-labile) is distinguished. In the course of the later stages of tumour growth the pH distribution gradually changes, more of the pH 3·5 enzyme appears, and further the ratio between the extinction maxima at 3·5 and 4·5 is quite

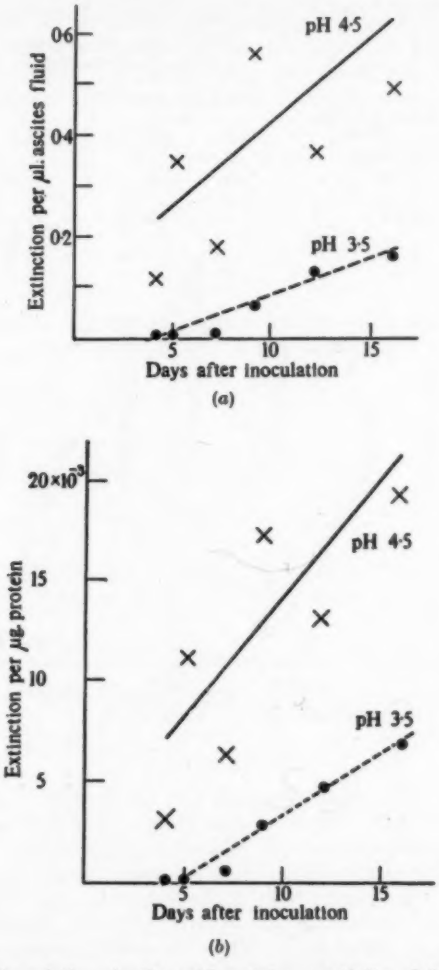

FIG. 6.—The observed variations in the acid proteinase activity of fresh cell-free ascites fluid at the pH optima 3·5 and 4·5. Each figure obtained from three pooled samples. Activities expressed per μl. fluid (6, A) as well as per μg. protein content (6, B).

different as compared with that in the blood plasma. Fig. 6, A and B, demonstrate the continuous increase in both activities in the later course of ascites tumour growth. The pH 3·5 enzyme in the ascites fluid does not reach the corresponding

plasma level of tumour-bearing mice at the end of tumour growth (Ottoson and Sylvén, 1959).

TABLE II.—*Serial Data on the Dipeptidase and Proteinase Activity per µl. Ascites Fluid Pooled from 3 Inbred Male Mice*

Age of tumour (in days)	Dipeptidase activity (µl. 0·1 M NaOH per µl. fluid)	Proteinase activity Extinction increase per µl. fluid			
		Cysteine added		Cysteine not added	
		pH 3·5 E	pH 4·5 E	pH 3·5 E	pH 4·5 E
4	0·53	0·01	0·12	0·01	0·08
5	—	0·00	0·32	—	—
7	0·43	0·01	0·18	0·03	0·14
9	0·31	0·07	0·58	0·08	0·52
12	0·74	0·14	0·36	0·15	0·30
16	1·8	0·17	0·48	0·22	0·54

TABLE III.—*Serial Data on the Dipeptidase and Proteinase Activity per Protein Content in Ascites Fluid Pooled from 3 Inbred Male Mice*

Age of tumour (in days)	Per cent protein content	Dipeptidase activity (µl. 0·1 M NaOH per mg. protein)	Proteinase activity Extinction increase per mg. protein			
			Cysteine added		Cysteine not added	
			pH 3·5 E	pH 4·5 E	pH 3·5 E	pH 4·5 E
4	4·7	5·4	0·2	3·0	0·3	1·8
5	3·9	—	0·0	11·0	—	—
7	2·9	14·7	0·5	6·2	1·1	4·6
9	3·4	9·2	2·3	17·2	2·5	15·8
12	2·8	44·6	4·8	13·0	5·2	10·8
16	2·5	72·0	6·7	19·2	8·5	21·0

So far all activities have been expressed per µl. ascites fluid and plasma. The observed increases in enzymic activity are still more marked when correlated to the protein content of the compartments (Table III).

DISCUSSION

The observed enzymic data illustrate the net results of a complex series of transport and permeability events *in vivo*, which seem difficult to distinguish in detail. Since the enzymes are unstable and are possibly subject to different degrees of dilution and inactivation in all the tissue compartments concerned the conclusions are necessarily vague, and have to be supported by other more direct and independent data. The discussion will mainly be based on the enzymic activities per unit volume as is usual in problems involving transport stable markers. Additional information may be obtained by comparing the enzymic activities on a per-protein basis.

It is further assumed that there is a rapid exchange of water and a slower exchange of protein constituents between the ascites fluid and blood mainly via the lymphatic pathways (Courtice and Steinbeck, 1950; Prentice, Siri and Joiner, 1952; Abdou, Reinhardt and Tarver, 1952; McKee *et al.*, 1952; Berson and Yalow, 1954; and Schoenberger *et al.*, 1956). It is further presupposed that the

ascites fluid originates from the blood serum (Straube, 1958), but also that certain ionic and enzymic contributions may appear from the interstitial fluid of the peritoneal walls as well as from the intracellular compartment of the peritoneal cells, normal as well as tumorous ones.

With reference to the exchange of proteins between the blood and ascites fluid present data seem to justify the conclusion that there is a " restricted diffusion " of globulins into the peritoneal cavity leading to the observed shift in the A/G ratio. This might indicate a certain " sieving-effect " on the part of the capillary membrane.

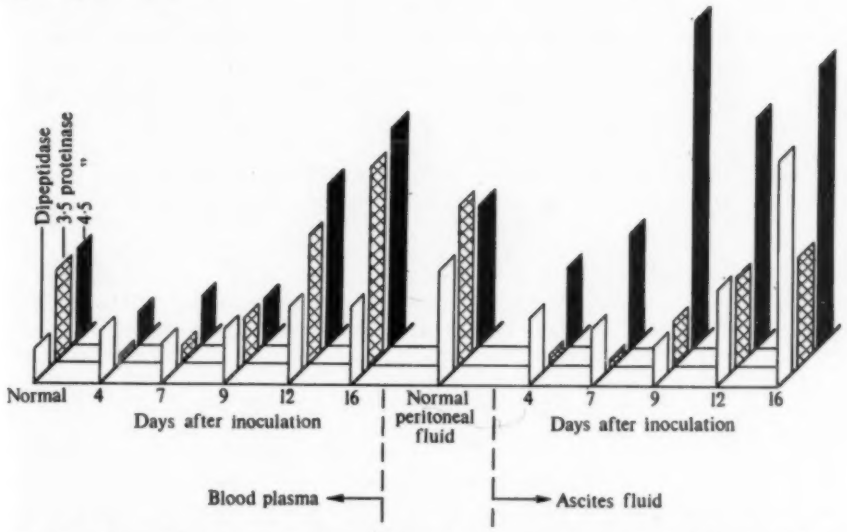

FIG. 7.—Comparison between the enzymal activities expressed per μl. fluid in the fluid compartments under question. The plasma data (Ottoson and Sylvén, 1959) are obtained on blood samples from the same mice as used in this report. The data on normal peritoneal fluid will be published elsewhere.

The relative enzymic activities of the fluid compartments under question are for comparison compiled in Fig. 7. According to previous concepts it may be tentatively assumed that the labile extracellular dipeptidase activity is associated with a relatively small protein molecule of a size close to the albumins, while the pH 4·5 proteinase activity most likely resides in a larger molecule possibly of the size of the globulins.

The dipeptidase concentration

In the course of the ascites tumour growth the plasma concentration of total dipeptidase activity increased to about twice the normal level (Ottoson and Sylvén, 1959). In the same mice the peptidase activity of the ascites fluid at first, before the 12th day after inoculation, had a similar concentration (Table II), but then rose to a maximum level of about three times that of the corresponding blood plasma activity, and twice that of the normal intraperitoneal fluid (Fig. 7).

The ascites tumour cells had a very high content of peptidases. The results can be explained by the assumption that peptidases are diffusing from the tumour cells into the ascitic fluid and then transported to the blood. The relative balance between the ascites fluid and blood plasma concentration during the first part of tumour growth might signify that the exchange is more rapid during this time (cf. Berson and Yalow, 1954 ; Straube, 1958) while possibly a relative retention of peptidases becomes more evident later on. Independent *in vitro* data (Sylvén, unpublished results) clearly show that the tumour cells are releasing peptidases at a high rate.

The concentration of acid proteinases

The proteinase pattern presents a complicated picture. During the first part of the ascites tumour growth most of the acid proteinase activity was abolished both in the blood plasma (Ottoson and Sylvén, 1959) and in the ascites fluid (Fig. 7). It is suggested that this may be due to an increased concentration of acid serum mucoproteins acting as proteinase inhibitiors (cf. above). The pH 3·5 enzyme certainly has some source other than the ascites tumour cells, since the latter do not contain this enzyme (Fig 4). The enzyme on the other hand is present in normal peritoneal fluid (unpublished results), although its actual activity level there is lower than in normal mouse plasma. It is possible that the pH 3·5 enzyme is derived from the stomach mucosa according to Mirsky *et al.* (1952). The present data do not give reliable information as to the actual concentrations of this enzyme due to the apparent presence of an inhibitor, and therefore, further discussion is postponed.

The pH 4·5 proteinase is present in the ascites tumour cells (Fig. 4). Also this enzyme seems to be influenced to some extent by the proteinase inhibitor in the ascites fluid during the first 5 to 7 days of tumour growth. In some series of mice the pH 4·5 enzyme shows a moderate activity in the ascites fluid at the 5th to 7th day after inoculation (Fig. 5). The activity then increases to a high level in the blood plasma as well as in the ascites fluid, although the activity in the latter compartment remains somewhat higher. On the assumption that the inhibitor concentration is of the same magnitude in both compartments, it appears probable that a transport of this enzyme occurs from the cells via the ascites fluid to the blood.

The fate of the peptidases and proteinases possibly transferred to the blood stream is not known ; a certain degree of inactivation and/or excretion is assumed to occur. The peptidases are for instance extremely sensitive, and may as well as the proteinases become excreted similar to its pepsin counterpart. The relative ratio in plasma between the extinctions of the pH 3·5 and the 4·5 proteinase activity leaves no indication as to the fate of the pH 4·5 plasma enzyme during the later part of tumour growth.

It will be noted that the observed differences in enzymic activities per volume would become increased if the data are recalculated per protein content of the different compartments (Table III). Since this may not be fully justified with a view to the transport problems involved, the authors have omitted to consider this set of data in detail.

The present results do not allow us to consider to what extent a selective retention of enzymes might contribute to the observed increases of enzymic

activity in the ascites compartment. The determinations are only an indication of the actual activities. Since uncontrolled factors are involved and in particular the presence of a proteinase inhibitor is assumed, the observed activity data do not represent the total enzyme contents, and cannot serve as indicators of the amount of blood proteins filtering through from the serum. Present data are therefore only circumstantial evidence in favour of the view that tumour cells, as well as normal cells, release enzymes to different extents into the extracellular fluid.

The reported *in vivo* experiments, however, are insufficient to explain the factors and environmental conditions responsible for the marked enzymic increase at the end of the ascites tumour growth. It is not known whether this increase is associated with an increased rate of cell death on the part of the tumour cells. Ács and Straub (1954) described a very marked increase in the oncentration of potassium ions in the ascites tumour fluid. This can also be interpreted as an indication of a release of intracellular K ions into the extracellular space. The increase in the K ion concentration was noted already very early during tumour growth, and did not seem to have a close correlation with the frequency of cell death.

The report illustrates the difficulties involved in the interpretation of data derived under *in vivo* conditions, where large differences in enzymic activity are required before significant results can be obtained on a per-volume basis.

SUMMARY

Quantitative assays were performed of the dipeptidase activity, against alanylglycine, and proteinase activity between pH 3 to 6, using urea-denatured haemoglobin as a substrate, in the cell-free ascites fluid of inbred male mice inoculated with a standard dose of the hyperdiploid Ehrlich ascites tumour. Comparison was made with previous serial assays, performed in the same mice, on the corresponding activities of blood plasma.

The quantitative growth characteristics of this ascites tumour line are described. The protein concentration of the cell-free ascites fluid descreased to a figure of about 2·5 per cent at the end of tumour growth. The changes in both the absolute and relative distribution of albumins and globulins in the blood plasma and ascites fluid of tumour-bearing mice were determined by means of electrophoresis.

The dipeptidase activity of the ascites fluid showed a continuous increase in the course of tumour growth, and reached shortly before the animals died a level of about three times that observed in the corresponding blood plasma. The pH 3·5 and 4·5 proteinases, normally present in blood plasma, were also demonstrable in the normal extracellular peritoneal fluid. The pH 3·5 proteinase activity was almost abolished in the early ascites fluid as well as in the plasma of the same mice. The pH 4·5 activity was simultaneously somewhat depressed. The pH 3·5 proteinase activity later on reappeared in both compartments, but only reached a level of about half the activity of the corresponding blood plasma. The pH 4·5 proteinase activity is similar to the intracellular cathepsins. This reached a high level in the ascites fluid constituting its main proteinase in the acid pH range. During the same time this activity was increased to twice the normal level in the blood plasma.

The conclusion is reached that, most likely, the increase in dipeptidase and pH 4·5 proteinase activity in the ascites fluid originate by release of enzymal proteins from intraperitoneal cells. Other independent data to be reported elsewhere support the view that most of the released enzymes have leaked out from the tumour cells.

The results are so far only valid in the case of ascites tumours; the plasma activities in mice with solid transplanted tumours remain to be studied.

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REFERENCES

ABDOU, I. A., REINHARDT, W. O. AND TARVER, H.—(1952) *J. biol. Chem.*, **194**, 15.
ÁCS, G. AND STRAUB, F. B.—(1954) *Acta Physiol. hung.*, **6**, 253.
BAYREUTHER, K.—(1952) *Z. Naturf.*, **7**, 554.
BENSON, J. A., KIM, K. S. AND BOLLMANN, J. L.—(1955) *Amer. J. Physiol.*, **182**, 217.
BERSON, S. A. AND YALOW, R. S.—(1954) *J. clin. invest.*, **33**, 377.
BOSCH, L.—(1958) *Biochim. biophys. Acta*, **30**, 444.
BRAUN-FALCO, O.—(1957) *J. Histo- Cytochem.*, **5**, 94.
BURSTONE, M. S.—(1956) *J. nat. Cancer Inst.*, **10**, 1149.
CHRISTENSEN, H. N. AND RIGGS, T. R.—(1952) *J. biol. Chem.*, **194**, 57.
COURTICE, F. C. AND STEINBECK, A. W.—(1950) *Aust. J. exp. Biol. med. Sci.*, **28**, 161.
DOYLE, W. L.—(1955) *J. Biophys. Biochim. Cytol.*, **1**, 221.
FEINSTEIN, R. N. AND BALLIN, J. C.—(1953) *Cancer Res.*, **13**, 780.
FLEISHER, G. A. AND BUTT, H. R.—(1953) *J. clin. Invest.*, **32**, 674.
FRUTON, J. S.—(1946) *J. biol. Chem.*, **166**, 721.
GRASSMANN, W. AND HEYDE, W.—(1929) *Z. physiol. Chem.*, **183**, 32.
HAUSCHKA, T. S., GRINNEL, S. T., RÉVÉSZ, L. AND KLEIN, G.—(1957) *J. nat. Cancer Inst.*, **19**, 13.
ISHINO, K.—(1935/38) *Arb. anat. Inst. Univ. Kyoto*, 5–7, 68.
KUN, E., TALALAY, P. AND WILLIAMS-ASHMAN, H. G.—(1951) *Cancer Res.*, **11**, 855.
LEDOUX, L. AND REVELL, S. H.—(1955) *Biochim. biophys. Acta*, **18**, 416.
LIBENSON, L. AND JENA, M.—(1957) *Cancer*, **10**, 1004.
MALMGREN, H., SYLVÉN, B. AND RÉVÉSZ, L.—(1955) *Brit. J. Cancer*, **9**, 473.
MCKEE, F. W., YULIE, C. L., LAMSON, B. G. AND WHIPPLE, G. H.—(1952) *J. exp. Med.*, **95**, 161.
MIRSKY, A., FUTTERMAN, P., KAPLAN, S. AND BROH-KAHN, R. H.—(1952) *J. Lab clin. Med.*, **40**, 17.
OSATO, S.—(1921) *Tohoku J. exp. Med.*, **2**, 514.
OTTOSON, R. AND SYLVÉN, B.—(1959) *Arch. Biochem. Biophys.* (in press).
PRENTICE, T. C., SIRI, W. AND JOINER, E. E.—(1952) *Amer. J. Med.*, **13**, 668.
RÉVÉSZ, L. AND KLEIN, G.—(1954) *J. nat. Cancer Inst.*, **15**, 253.
SCHADE, A. L.—(1953) *Biochim. biophys. Acta*, **12**, 163.
SCHOENBERGER, J. A., KROLL, G., ECKERT, E. L. AND KARK, R. M.—(1956) *J. Lab. clin. Med.*, **47**, 227.
STRAUBE, R. L.—(1958) *Cancer Res.*, **18**, 57.
SYLVÉN, B.—(1945) *Acta radiol. Stockh.*, Suppl. 59, "Ester Sulphuric Acids of High Molecular Weight and Mast Cells in Mesenchymal Tumors.—(1954) *Acta Un. int. Cancr.*, **10**, 169.

SYLVÉN, B. AND MALMGREN, H.—(1957) *Acta radiol. Stockh.*, Suppl. 154, "The Histological Distribution of Proteinase and Peptidase Activity in Solid Tumor Transplants"

WARBURG, O. AND CHRISTIAN, W.—(1943) *Biochem. Z.*, **314**, 399.

WU, R. AND RACKER, E.—(1958) *Fed. Proc.*, **17**, 239.

YOFFEY, J. M. AND COURTICE, F. C.—(1956) "Lymphatics, Lymph and Lymphoid Tissue". 2nd Ed. London (E. Arnold Publ. Ltd.).

MITOCHONDRIA OF THE EHRLICH ASCITES TUMOUR CELL —SWELLING CHARACTERISTICS

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THE contention of Warburg (1956) that tumour cell mitochondria are irreparably damaged, has prompted numerous comparisons between the properties of normal and tumour cell mitochondria. The isolation of Ehrlich ascites tumour mitochondria and studies of their oxidative phosphorylation with a variety of substrates were reported in a previous communication from these laboratories (Hawtrey and Silk, 1959). The present report concerns further studies, but of a physical nature—namely, the swelling characteristics of both respiring and non-respiring Ehrlich ascites mitochondria in a variety of media. The characteristics have been compared with those of mitochondria from normal cells and from other tumour types under similar conditions.

A comparison between the swelling characteristics of normal and tumour cell mitochondria provides information concerning the influence of intracellular and extracellular fluid environment on mitochondrial functioning. Studies in these laboratories have demonstrated that the fluid environment of tumour cells is of paramount importance as the medium through which X-radiation acts "indirectly" on respiratory metabolism (Silk, Hawtrey and Macintosh, 1958). The environmental "indirect" action of X-radiation is at present being studied using Ehrlich tumour mitochondria as test material in place of whole cells. Accordingly it became necessary to investigate the influence of environmental medium pH and composition on the physical characteristics of Ehrlich tumour mitochondria. Such physical studies are of importance because of the possible influence of swelling and shrinkage on the structural arrangement of enzymes necessary for chemical functioning of the mitochondria (Tedeschi and Harris, 1958).

MATERIALS AND METHODS

Ehrlich ascites tumour.—This tumour was kindly supplied by Dr. K. Sugiura of the Sloan-Kettering Institute, New York, and has been maintained in Swiss albino mice which were used between the 9th and 12th day after inoculation.

Hexokinase (General Biochemicals Inc., Ohio, U.S.A.) when assayed according to Somogyi (1952) and Crane and Sols (1955) was found to have an activity of approximately 150 units per mg.

Cytochrome c was used as a 1 per cent solution in isotonic saline as supplied by General Biochemicals Inc., and was found to contain $5{\cdot}87 \times 10^{-7}$ moles per ml. when assayed according to Umbreit, Burris and Stauffer (1957).

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Adenosine-5-phosphoric acid (*AMP*) and *Adenosine diphosphate* (*ADP*) were used as supplied by General Biochemicals Inc., and by the H.M. Chemical Co. of Los Angeles, California, respectively.

Adenosine triphosphate (*ATP*) was used as the tetra sodium salt supplied by General Biochemicals Inc.; and assayed as 85 per cent pure by the method of Crane and Sols (1955).

Diphosphopyridine nucleotide (*DPN*) was obtained from General Biochemicals Inc., and assayed as 85 per cent pure by a modification of the method of Brodie (1955) using 10 mg. of sodium dithionite per 1·0 ml. of 0·2 M dibasic sodium phosphate as the reducing solution.

All other substrates and chemicals used were of Analytical Reagent grade.

Isolation of mitochondria

Mitochondria were isolated by the method of Hawtrey and Silk (1959). All operations were carried out at 0–4° C. Saline washed ascites cells were allowed to swell for 15–20 minutes in hypotonic ($\frac{1}{4}$ strength) Ringer phosphate solution, and subsequently disrupted into 8–10 volumes of pure water using a loose fitting Dounce homogeniser (Blaessig Glass Specialities, Rochester, New York, U.S.A.) for exactly 3·0 minutes. Immediately after lysis, concentrated sucrose solution was added to the homogenate to give the usual 0·25 M sucrose suspension from which the mitochondria were isolated by differential centrifugation. Nuclei and cell debris were removed at 600–700 g. for 20 minutes, and the supernatant centrifuged at 12,000–13,000 g. for 10 minutes to sediment the mitochondrial fraction. The mitochondria were washed twice until free of " fluffy " layer, and were then re-suspended in 0·25 M sucrose (approximately 5–6 ml. per g. wet weight) equivalent to a concentration of 4–6 mg. mitochondrial protein per ml. The pH of this final suspension was 6·9.

Mitochondrial protein content was determined according to the biuret method of Cleland and Slater (1953) using an ovalbumin standard.

Media for determination of mitochondrial swelling

(a) *Non-respiring mitochondria.*—The medium is indicated for each experiment.

(b) *Respiring mitochondria.*—The following medium was used: 0·045 M sodium potassium phosphate buffer (pH 7·4); 0·015 M KF; 0·008 M $MgCl_2$; 0·0025 M ADP; 0·001 M AMP; 0·002 M EDTA (ethylene diamine tetra acetic acid); 0·01 M d-glucose; 0·032 M sucrose; $2{\cdot}4 \times 10^{-4}$ M DPN; $9{\cdot}8 \times 10^{-6}$ M cytochrome c; 1–3 mg. hexokinase (crystalline); 0·007 M substrate. (Media with α-ketoglutarate as substrate contained 0·02 M malonate in addition. AMP, ADP and all substrates were neutralised to pH 7·4 with KOH and stored at −30° C. before use.)

For experiments in which co-factors or the glucose-hexokinase phosphate acceptor system were omitted from the medium, 0·25 M sucrose was used to make up the necessary volume.

Measurement of mitochondrial swelling

The change in optical density of a dilute suspension of mitochondria was measured according to the method of Cleland (1952).

Measured volumes (0·15 ml.) of the mitochondrial suspension in 0·25 M sucrose

(containing 4–6 mg. protein/ml.) were added to 2·85 ml. of the medium in which swelling was to be determined. After thorough mixing, the optical density at 520 mμ was read in a Model DU 2400 Beckman Spectrophotometer against a blank containing the medium under examination plus 0·25 M sucrose in place of the mitochondrial suspension. Values are expressed as Specific Extinction coefficients

$$\left(E\,\frac{1\%}{1\ \text{cm}}\right)$$

for suspensions containing 1 per cent of mitochondrial protein.

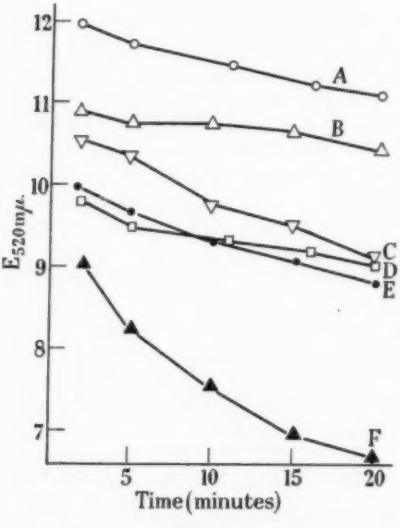

FIG. 1.

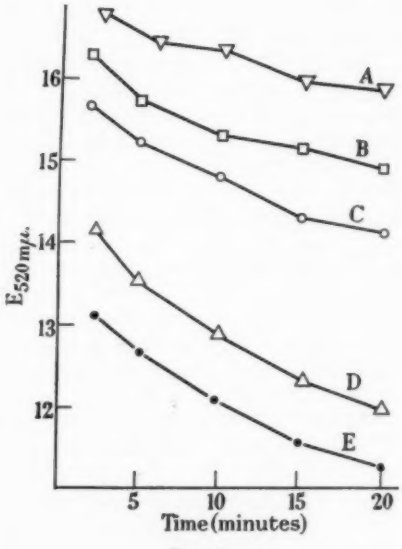

FIG. 2.

FIG. 1.—Swelling of non-respiring mitochondria in unbuffered sucrose solutions. A, 0·25 M sucrose + 0·01 M EDTA (pH 7·4); B, 0·25 M sucrose; C, 0·44 M sucrose; D, distilled water; E, 0·80 M sucrose; F, 0·60 M sucrose.

FIG. 2.—Swelling of non-respiring mitochondria in sucrose solutions buffered to pH 7·4 by addition of 0·05 M tris (hydroxymethyl)-aminomethane-acetate. A, 0·25 M sucrose + 0·01 M EDTA; B, 0·25 M sucrose; C, 0·44 M sucrose; D, 0·60 M sucrose; E, 0·80 M sucrose.

RESULTS

Each family of curves shown in Fig. 1–9 was obtained using a single mitochondrial preparation. A control determination of Q_{O_2} value and P : O ratio with succinate as substrate was carried out on each batch of mitochondria, to ensure their functional integrity for use in swelling experiments. All the preparations used showed a satisfactory level of oxidative phosphorylation activity with succinate (cf. Hawtrey and Silk, 1959). Q_{O_2} values lay within the range 85–125 and P : O ratios within the range 1·1–1·4. (Q_{O_2} values are expressed as μl. O_2 uptake per mg. protein per hour.)

Swelling of non-respiring mitochondria

The curves in Fig. 1 show that in unbuffered solutions the swelling of non-respiring mitochondria is minimal in 0·25 M sucrose which was used in the isolation procedure. Swelling of mitochondria was also negligible in water which was used as suspending medium for initial lysis of the ascites cells. No agglutination of mitochondria was observed in aqueous suspension. A certain amount of mitochondrial swelling was observed in sucrose solutions above 0·25 M in concentration. The presence of 0·01 M EDTA caused a slight increase in the swelling observed in 0·25 M sucrose. The swelling of non-respiring mitochondria in sucrose solutions buffered to pH 7·4 with tris-(hydroxymethyl)-aminomethane-acetate (Fig. 2) was on the whole slightly more than in unbuffered solutions (Fig. 1). As with

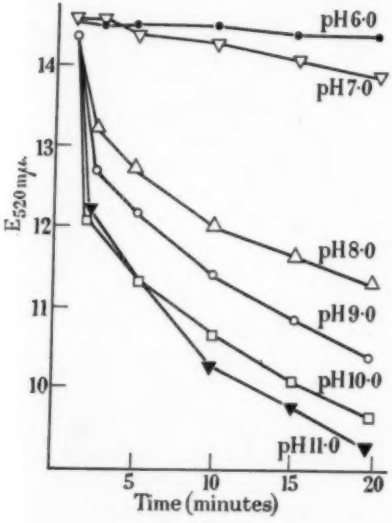

FIG. 3.

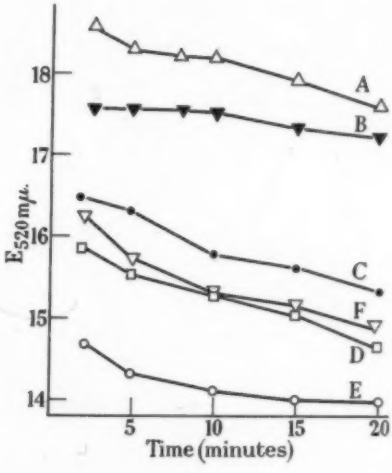

FIG. 4.

FIG. 3.—The effect of pH on the swelling of non-respiring mitochondria. Mitochondrial suspension in 0·25 M sucrose (0·15 ml.) was added to 2·85 ml. of 0·25 M sucrose buffered with 0·05 M tris-acetate to the pH indicated for each curve. At pH values below 6·0, agglutination of the mitochondria occurred.

FIG. 4.—Swelling of non-respiring mitochondria in 0·25 M sucrose solutions buffered with 0·05 M tris-acetate (pH 7·4), and containing added co-factors: A, 0·001 M ATP; B. 0·001 M DPN; C, 0·001 M AMP; D, 0·001 M ATP + 0·001 M DPN; E, 0·001 M ADP; F, control.

unbuffered solutions the swelling was least in 0·25 M sucrose, but the increase of swelling on addition of EDTA to unbuffered solutions was not observed in buffered solutions. Whereas with unbuffered solutions, swelling was greatest in 0·60 M sucrose and less in 0·80 M (Fig. 1), in buffered solutions swelling was comparable in both concentrations of sucrose (Fig. 2).

Non-respiring mitochondria did not swell in sucrose solution buffered to pH 6·0 with tris-acetate, but swelling was observed at pH 7·0 and was found to be

increased at successively higher pH values (Fig. 3). Agglutination of the mitochondria occurred below pH 6·0.

The swelling of non-respiring mitochondria in 0·25 M sucrose buffered to pH 7·4 with tris-acetate was considerably decreased by addition of 0·001 M DPN to the medium (Fig. 4). Addition of ADP caused a slight inhibition of swelling, while AMP and ATP were without effect. The protective action of DPN was nullified when ATP was also present in solution (Fig. 4).

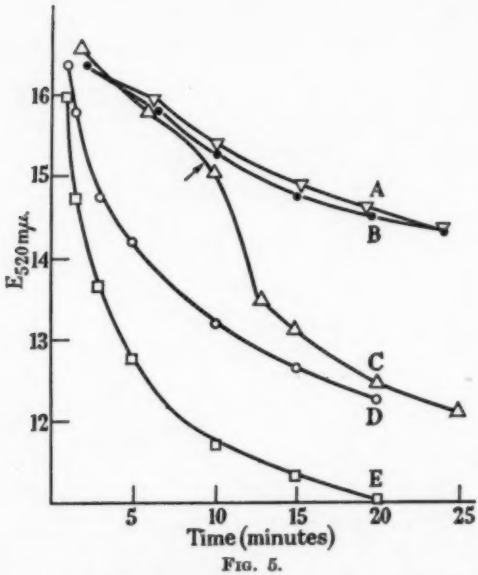

FIG. 5.

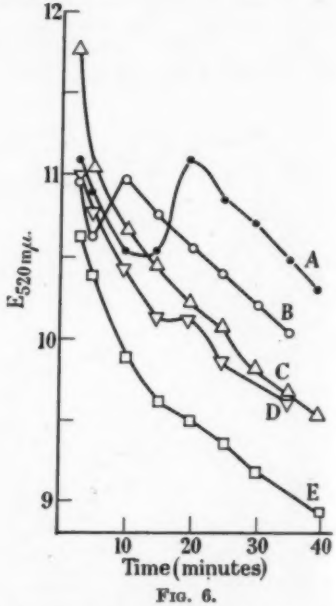

FIG. 6.

FIG. 5.—Swelling of non-respiring mitochondria in 0·25 M sucrose solutions buffered with 0·05 M tris-acetate (pH 7·4) and containing various additions: A, $1{\cdot}2 \times 10^{-4}$ M 2:4-dinitrophenol (DNP); B, control; C, 3×10^{-5} M L-thyroxine added as indicated (↓); D, 3×10^{-5} M L-thyroxine; E, 0·01 M K, Na phosphate + 0·001 M potassium succinate.

FIG. 6.—Swelling of respiring mitochondria in phosphorylating medium (see text) with various substrates. A, 0·01 M succinate; B, 0·0065 M ascorbate; C, 0·01 M DL-β-hydroxybutyrate; D, 0·0066 M L-glutamate; E, no substrate.

The swelling of non-respiring mitochondria in 0·25 M sucrose buffered to pH 7·4 with tris-acetate was not affected by addition of the uncoupling agent DNP (2:4 dinitrophenol). On the other hand addition of thyroxine *ab initio* or at a subsequent stage in the experiment, caused a marked increase in swelling (Fig. 5) but only with 6 out of 8 mitochondrial preparations examined. Rapid swelling of non-respiring mitochondria in buffered sucrose was observed on addition of succinate and phosphate to the medium (Fig. 5).

Swelling of respiring mitochondria

Mitochondria in the phosphorylating medium without added substrate underwent the same degree of swelling as observed for non-respiring mitochondria

in 0·25 M sucrose. However, in the presence of certain added substrates shrinkage occurred followed by a gradual degree of swelling (Fig. 6). Succinate caused the maximum shrinkage of swollen mitochondria, while ascorbate was practically as effective though more rapid in its action. Addition of L-glutamate caused only mild shrinkage of the mitochondria while addition of β-hydroxybutyrate was without effect on the swelling (Fig. 6).

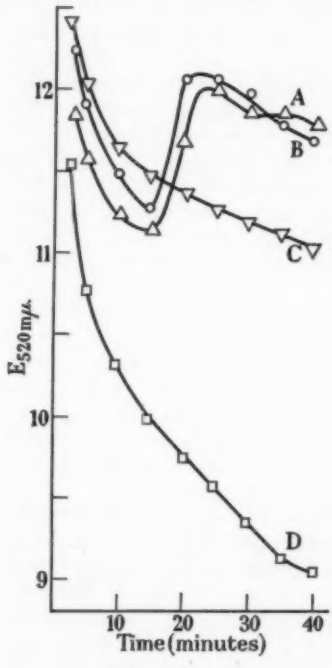

FIG. 7.

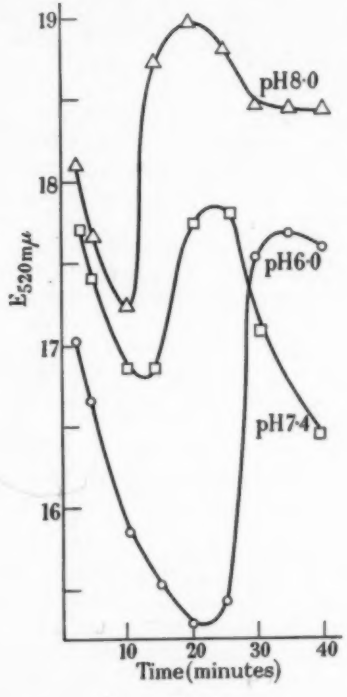

FIG. 8.

Fig. 7.—Swelling of respiring mitochondria in phosphorylating medium (see text) with succinate as substrate. Effect of removing co-factors from the medium. A, no hexokinase; B, control; C, no cytochrome c or hexokinase; D, no cytochrome c.

FIG. 8.—Swelling of respiring mitochondria in phosphorylating media at various pH values with succinate as substrate. pH 8·0: medium as in text but with (0·005 M K,Na phosphate buffer + 0·040 M tris-acetate buffer) in place of the 0·045 M K,Na phosphate buffer specified. pH 7·4: medium as in text. pH 6·5: medium as specified for pH 8·0. Agglutination of mitochondria occurred below pH 6·0, and heavy precipitates formed in the medium above pH 8·0.

The shrinkage of swollen mitochondria in phosphorylating medium with succinate as substrate, was unchanged by removal of the hexokinase-phosphate esterifying system from the medium (Fig. 7). On the other hand removal of cytochrome c from the medium resulted in rapid swelling and complete failure

of the mitochondria to undergo shrinkage. Removal of cytochrome c and hexokinase together also resulted in failure to undergo shrinkage, but the degree of initial mitochrondrial swelling was less than in the case where only cytochrome c was removed from the medium (Fig. 7).

The effect of medium pH on the shrinkage of swollen mitochondria in phosphorylating medium with succinate as substrate, was studied in a series of experiments. Shrinkage took place after 10 minutes at pH 8·0, after 12 minutes at pH 7·4 but only after 22 minutes at pH 6·0. Agglutination of mitochondria occurred below pH 6·0, while heavy precipitates formed in the medium above pH 8·0 (Fig. 8).

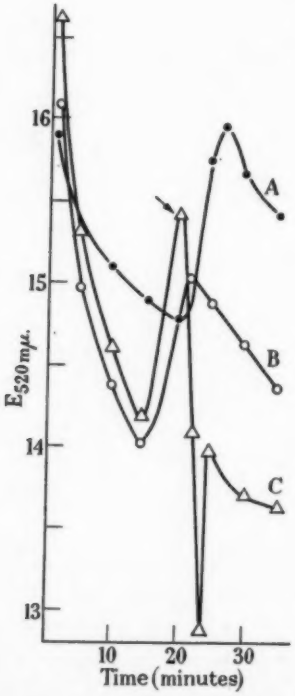

FIG. 9.—Effect of thyroxine on swelling of respiring mitochondria in phosphorylating medium (see text) with succinate as substrate. A, 3×10^{-5} M thyroxine present initially; B, no thyroxine; C, 3×10^{-5} M thyroxine added as indicated (↘).

Shrinkage of swollen mitochondria in phosphorylating medium with succinate as substrate, was also observed in the presence of 3×10^{-5} M thyroxine, although the initial swelling was less than in a medium without thyroxine. Addition of 3×10^{-5} M thyroxine to the medium immediately after shrinkage of the respiring mitochondria had taken place, caused instantaneous and marked swelling followed by rapid shrinkage and a subsequently more gradual rate of swelling (Fig. 9).

DISCUSSION

Swelling of non-respiring Ehrlich tumour mitochondria was minimal in water and in 0·25 M sucrose used for the isolation procedure. The method of isolation involved brief contact of the mitochondria with water over a period of 3·0 minutes during initial lysis of the ascites cells, but it has been shown that such treatment does not impair their capacity for oxidative phosphorylation (Hawtrey and Silk, 1959). Concentrated sucrose solution is added to the homogenate immediately after the 3·0 minute lysis period and this, in all probability, completely reverses any swelling which may occur during contact with the water (Tedeschi and Harris, 1958).

The observation that initial $E_{520\ m\mu}$ values for a single batch of mitochondria are different in sucrose solutions of varying osmolarity (cf. Fig. 1 and 2) is attributable to differences in refractive index of the suspending media (Tedeschi and Harris, 1958). The same divergence of initial $E_{520\ m\mu}$ values in varying sucrose concentrations was found by Mutolo and Abrignani (1957). In a later paper by Mutolo and Abrignani (1958) curves are shown for tumour mitochondria which have the same initial $E_{520\ m\mu}$ value for a single sucrose concentration. Our results also show that in a single medium the swelling curves for Ehrlich mitochondria tend towards a common origin.

The observation that non-respiring tumour mitochondria show an increased degree of swelling with increasing sucrose molarity in buffered or unbuffered solution, is in contrast to the finding of Lehninger (1956) that rat liver mitochondria exhibit least swelling in hypertonic sucrose. The apparent increase of swelling with increasing solution osmolarity is in itself not easily explicable, but may be due to a gradual change in internal refractive index of the mitochondrial particles (cf. Tedeschi and Harris, 1958, and Barer, Ross and Tkaczyk, 1953).

Failure of EDTA to prevent the swelling of non-respiring Ehrlich mitochondria in unbuffered sucrose is in keeping with the results of Mutolo and Abrignani (1957) who found that it tended to promote the swelling of Walker 256 carcinosarcoma mitochondria. EDTA effectively prevents the swelling of rat liver mitochondria (Mutolo and Abrignani, 1957), and also stabilises the oxidative activity of heart muscle sarcosomes (Cleland and Slater, 1952).

The increase in swelling of non-respiring Ehrlich mitochondria in 0·25 M sucrose at successively higher pH values, parallels the results of Witter and Cottone (1956) using rat liver mitochondria. Liver and Ehrlich tumour mitochondria show least swelling at pH 6·0, and below pH 6·0 rapid agglutination of both types occurs (Gamble, 1957).

The swelling of non-respiring Ehrlich mitochondria in 0·25 M sucrose buffered to pH 7·4 was not reversed on addition of ATP, ADP, AMP, DPN or ATP + DPN to the medium. DPN and to a lesser extent ADP were however effective in decreasing the rate of swelling, although the protective action of DPN was nullified when ATP was also present in solution. By contrast Fonnesu and Davies (1956) and Dickens and Salmony (1956) found the swelling of liver mitochondria to be reversed by addition of ATP. A similar effect was shown by Chappell and Perry (1954) using heart muscle sarcosomes. Failure of ATP and DPN to reverse the swelling of Ehrlich ascites cell mitochondria, may be due to the high ATP-ase and DPN-ase activities of tumour mitochondria as distinct from those of normal

cells. No fluoride or nicotinamide were added to inhibit these actions in the media used to examine the Ehrlich mitochondria.

The uncoupling agent 2 : 4 dinitrophenol (DNP) was without effect on the swelling of non-respiring Ehrlich mitochondria in 0·25 M sucrose buffered to pH 7·4. On the other hand, Lehninger (1956) observed that DNP effectively prevented the swelling of rat liver mitochondria. Thyroxine, another uncoupling agent, caused a rapid increase in the swelling of non-respiring Ehrlich mitochondria which parallels the findings of Dickens and Salmony (1956) and Lehninger (1956) with non-respiring rat liver mitochondria, and is in contrast to the findings of Emmelot and Bos (1957) with rat hepatoma mitochondria. However, the effect of thyroxine on normal cell mitochondria is apparently variable. Tapley and Cooper (1956) found that rat liver and kidney mitochondria underwent swelling in the presence of thyroxine whereas those of the heart, testis and spleen did not.

Rapid swelling of non-respiring Ehrlich tumour mitochondria was observed in the presence of oxidizable substrate (succinate) and inorganic phosphate. This is analogous to the behaviour of rat liver mitochondria reported by Chappell and Greville (1958), and is in keeping with the statement of Witter and Cottone (1956) that succinate and phosphate cause a breakdown of organised mitochondrial structure. In a fortified phosphate medium but without substrate, Ehrlich mitochondria underwent the same degree of swelling as observed in 0·25 M sucrose buffered to pH 7·4.

The swelling of respiring Ehrlich mitochondria was studied in the same fortified incubation medium with a variety of substrates. Uninterrupted swelling was observed with L-glutamate and β-hydroxybutyrate, possibly due to the low quantity of DPN present, but with succinate and ascorbate an apparent reversal of swelling was observed. Moreover, this reversal occurred only in the presence of added cytochrome c and was unaffected by removal of the hexokinase trapping system. The possibility exists that two phenomena are involved simultaneously in the observation of swelling reversal at 520 mμ; (*a*) actual physical shrinkage of the mitochondria in the presence of cytochrome c, and (*b*) reduction of the external cytochrome c which could act as an electron acceptor if the conditions in the Beckman cuvette were anaerobic at the time when the reversal of swelling was observed. Part of the increased absorption at 520 mμ could therefore be due to the β-band of reduced cytochrome c. Blank experiments in which solid sodium dithionite was added to the cuvette at the start of the observed shrinkage, showed only 30 per cent of the increase in optical density usually found with succinate as substrate. Thus 30 per cent of the increase in optical density observed after 15 minutes with succinate and ascorbate may be due to reduction of the external cytochrome c, while 60 per cent may be due to actual shrinkage. Holton (1955) and Chance (1957) have shown that reduction of cytochromes in a mitochondrial suspension can be followed in the Beckman spectrophotometer.

Shrinkage of respiring mitochondria in the presence of succinate and cytochrome c was found to take place more rapidly at successively higher values of pH. Agglutination of the mitochondria occurred below pH 6·0 and above pH 8·0 heavy precipitates formed in the solution.

If added initially, thyroxine decreased the usual rate of swelling of respiring Ehrlich mitochondria, whereas the opposite effect was observed with non-respiring

mitochondria. However, if thyroxine was added when maximum shrinkage of respiring mitochondria had taken place, an immediate swelling of the particulates was observed. This was followed first by rapid shrinkage and then by a more gradual rate of swelling.

The physical characteristics of Ehrlich ascites cell mitochondria therefore appear to be similar to those reported for other tumour mitochondria, but differ in a number of respects from those of normal cell mitochondria. However, on the basis of the comparisons drawn it would not be possible to decide whether the observed differences might be generally characteristic of tumour cell mitochondria.

SUMMARY

1. The swelling characteristics of both respiring and non-respiring Ehrlich ascites cell mitochondria have been examined in a variety of media.

2. Swelling of non-respiring mitochondria is minimal in water and in 0·25 M sucrose and is not inhibited by addition of versene to the medium.

3. Swelling of non-respiring mitochondria increased with successive increases in pH value of the suspending medium. Agglutination of the mitochondria occurs below pH 6·0.

4. The swelling of non-respiring mitochondria cannot be reversed by addition of ATP, ADP, AMP, DPN or ATP + DPN to the medium. However, DPN and, to a lesser extent, ADP are effective in decreasing the rate of swelling.

5. Dinitrophenol is without effect on the swelling of non-respiring mitochondria in buffered sucrose. Thyroxine causes a rapid increase in swelling of non-respiring mitochondria.

6. Rapid swelling of non-respiring mitochondria occurs in the presence of oxidizable substrate (succinate) and inorganic phosphate.

7. Mitochondria allowed to respire with ascorbate and succinate as substrates apparently undergo a temporary reversal of swelling in the presence of added cytochrome c. The phenomenon is not observed with L-glutamate and β-hydroxybutyrate as substrates, possibly due to the low quantity of DPN present in the test medium.

8. The apparent reversal of mitochondrial swelling observed with succinate and ascorbate as substrates may be due in part to actual shrinkage and in part to reduction of external cytochrome c.

9. Shrinkage of respiring mitochondria in the presence of succinate and cytochrome c takes place more rapidly at higher values of medium pH. As with non-respiring mitochondria, agglutination occurs below pH 6·0.

10. Thyroxine added initially to respiring mitochondria decreases the usual rate of swelling. If added when maximum shrinkage of swollen mitochondria has occurred, thryoxine causes an immediate swelling followed by rapid shrinkage. This is followed again by a more gradual rate of swelling.

11. The swelling characteristics of Ehrlich tumour cell mitochondria have been compared with those reported for mitochondria from other tumour types and from normal cells.

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REFERENCES

BARER, R., ROSS, K. F. A. AND TKACZYK, S.—(1953) *Nature, Lond.*, **171**, 720.

BRODIE, A. F.—(1955) 'Methods in Enzymology,' **2**, 693. Eds. S. P. Colowick and N. O. Kaplan. New York (Academic Press Inc.).

CHANCE, B.—(1957) *Ibid.*, **4**, 273.

CHAPPELL, J. B. AND GREVILLE, G. D.—(1958) *Nature, Lond.*, **182**, 813.

Idem AND PERRY, S. V.—(1954) *Ibid.*, **173**, 1094.

CLELAND, K. W.—(1952) *Ibid.*, **170**, 497.

Idem AND SLATER, E. C.—(1952) *Ibid.*, **170**, 118.—(1953) *Biochem. J.*, **53**, 547.

CRANE, R. K. AND SOLS, A.—(1955) 'Methods in Enzymology,' **1**, 277. Eds. S. P. Colowick and N. O. Kaplan. New York (Academic Press Inc.).

DICKENS, F. AND SALMONY, D.—(1956) *Biochem. J.*, **64**, 645.

EMMELOT, P. AND BOS, C. J.—(1957) *Exp. Cell Res.* **12**, 191.

FONNESU, A. AND DAVIES, R. E.—(1956) *Biochem. J.*, **64**, 769.

GAMBLE, J. L.—(1957) *Biochim. biophys. Acta*, **23**, 306.

HAWTREY, A. O. AND SILK, M. H.—(1959) *Biochem. J.*, in the press.

HOLTON, F. A.—(1955) *Ibid.*, **61**, 46.

LEHNINGER, A. L.—(1956) 'Enzymes: Units of Biological Structure and Function'. Ed. O. H. Gaebler. New York (Academic Press Inc.), p. 217.

MUTOLO, V. AND ABRIGNANI, F.—(1957). *Brit. J. Cancer*, **11**, 590.—(1958) *Ibid.*, **12**, 285.

SILK, M. H., HAWTREY, A. O. AND MACINTOSH, I. J. C.—(1958) *Cancer Res.*, **18**, 1257.

SOMOGYI, M.—(1952) *J. biol. Chem.*, **195**, 19.

TAPLEY, D. F. AND COOPER, C.—(1956) *Nature, Lond.*, **178**, 1119.

TEDESCHI, H. AND HARRIS, D. L.—(1958) *Biochim. biophys. Acta*, **28**, 392.

UMBREIT, W. W., BURRIS, R. H. AND STAUFFER, J. F.—(1957) 'Manometric Techniques and Tissue Metabolism'. Minneapolis (Burgess Pub. Co.), p. 301.

WARBURG, O.—(1956) *Science*, **123**, 309.

WITTER, R. F. AND COTTONE, M. A.—(1956) *Biochim. biophys. Acta*, **22**, 364.